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IMMOBILIZED LACCASE BIOCATALYSTS AS A WAY TO IMPROVE DEGRADATION OF MICROPOLLUTANTS FROM WATER

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Orientador: Prof. Dra. Luciana Rocha Barros Gonçalves.

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To God.
To my parents, Tarcília e Carlos,
my brother, André,
and my husband, Lucas.

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“Siga sempre em frente. Sem sofrer.” (Bruna Pinheiro).

RESUMO

Nos últimos anos, desenvolver um biocatalisador ecológico para melhorar a qualidade da água surge como um dos estudos mais relevantes. Dentre os micropoluentes presentes na água estão o acetaminofeno e ácido mefenâmico, duas drogas muito utilizadas para tratar dores e febre pelo mundo que estão relacionadas com asma e aumento da toxicidade do sistema nervoso central. Biocatalisadores produzidos com lacase têm ganhado atenção para degradar esses poluentes. A lacase catalisa a oxidação de substratos fenólicos e possuem muitas aplicações, o que torna esta enzima adequada e atrativa para aplicações industriais. No entanto, as lacases solúveis têm sua disponibilidade diminuída devido à perda de estabilidade, alto custo de produção e não reutilização. Desta forma, a imobilização surge como uma forma de tornar essas potenciais aplicações uma realidade. Na primeira abordagem deste trabalho, a aminação química da lacase seguida de adsorção iônica em suportes catiônicos e aniônicos foi realizada a fim de melhorar as propriedades da enzima. O processo de aminação introduz grupos amino na superfície da proteína e podem ser controlados através do uso de diferentes concentrações de 1-etil-3-(3-dimetilaminopropil) carbodiimida (EDAC). Três quantidades de EDAC foram testadas (0.024 g, 0.048 g e 0.168 g), evidenciando que a estabilidade da lacase melhora à medida que mais grupos amino são introduzidos. Na segunda abordagem deste trabalho, foi realizado um estudo da imobilização *layer-by-layer* da lacase em quitosana ativada com genipina ou glutaraldeído. Desta forma, as multicamadas de lacase foram preparadas seguindo diferentes combinações de genipina e glutaraldeído para preparar a primeira e a segunda camadas de lacase. Essa técnica foi escolhida para aumentar a capacidade de carregamento do suporte, porém a adição de uma segunda camada nem sempre produziu biocatalisadores mais ativos. Finalmente, os biocatalisadores produzidos foram usados na degradação do acetaminofeno e do ácido mefenâmico. Os biocatalisadores de dupla camada revestido com genipina promoveram maior remoção dos contaminantes com 100% de degradação do ácido mefenâmico e de 66% do acetaminofeno, nas condições testadas.

Palavras-chave: Immobilização. Lacase. Aminação. *Layer-by-layer.* Adsorção

ABSTRACT

Over the last years, design a green and environmentally biocatalyst to improve water quality emerges as one of the most relevant studies nowadays. Among the micropollutants present in water are acetaminophen and mefenamic acid, two drugs widely used to treat pain and fever around the world. They are related to asthma and increased toxicity of the central nervous system. Laccase-based biocatalysts have gained attention to degrade micro-pollutants. Laccase catalyzes the oxidation of phenolic substrates and has many applications which make this enzyme suitable and attractive for industrial applications. However, soluble laccases have diminished availability due to loss of stability, high cost of production, and non-reusability. In this way, laccase immobilization emerges as a way to make these potential applications a reality. In the first approach of this work, chemical amination of laccase following by ionic adsorption on cationic and anionic supports was performed in order to improve the laccase properties. The amination process introduces amino groups on the enzyme surface and may be controlled by the 1-ethyl-3-(3-dimethylamino propyl) carbodiimide (EDAC) concentration. Three quantities of EDAC were tested (0.024 g, 0.048 g e 0.168 g), evidencing that the stability of the laccase improves as more amino groups are introduced. In the second approach of this work, a study of laccase layer-by-layer immobilization onto chitosan activated with genipin or glutaraldehyde was performed. In this way, laccase multilayers were prepared following different combinations of genipin and glutaraldehyde to prepare the first and second laccase layers. This technique was chosen to enhance the loading capacity of the support; however, the addition of a second layer did not always produce a more active biocatalyst. Finally, the biocatalysts produced were used in the degradation of acetaminophen and mefenamic acid. The genipin-coated double-layer biocatalysts promoted greater removal of contaminants with 100% degradation of mefenamic acid and 66% of acetaminophen, under the conditions tested.

Keywords: Immobilization. Laccase. Amination. Layer-by-layer. Adsorption.

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LIST OF ABBREVIATIONS AND ACRONYMS

EDAC	1-ethyl-3-(dimethylamino-propyl) carbodiimide
EDA	Ethylenediamine
DEAE	Diethylamino ethyl-agarose
MANAE	Monoaminoethyl–N–Ethyl-agarose
PL	Purified laccase
CL	Commercial laccase
A1	Purified enzyme aminated with 0,024g of EDAC
A2	Purified enzyme aminated with 0,072g of EDAC
A3	Purified enzyme aminated with 0,168g of EDAC
PLDEAE	Purified laccase immobilized in DEAE-agarose
PLMANAE	Purified laccase immobilized in MANAE-agarose
DA1	A1 immobilized in DEAE
DA2	A2 immobilized in DEAE
DA3	A3 immobilized in DEAE
SA1	A1 immobilized in sulfopropyl
SA2	A2 immobilized in sulfopropyl
SA3	A3 immobilized in sulfopropyl
GenLac	Chitosan-immobilized laccase activated with genipin
GluLac	Chitosan-immobilized laccase activated with glutaraldehyde
GenLacGlu	GenLac coated with glutaraldehyde
GluLacGlu	GluLac coated with glutaraldehyde
GenLacGen	GenLac coated with genipin
GluLacGen	GluLac coated with genipin
GenLacGluLac	Second layer of laccase immobilized in glutaraldehyde-coated GenLac
GluLacGluLac	Second layer of laccase immobilized in glutaraldehyde-coated GluLac
GenLacGenLac	Second layer of laccase immobilized in genipin-coated GenLac
GluLacGenLac	Second layer of laccase immobilized in genipin-coated GluLac

SUMMARY

Chapter 1

Introduction

1 INTRODUCTION

In recent years, water quality is one of the most relevant topics (BECKER et al., 2016; CHEN et al., 2018; UPADHYAY; SHRIVASTAVA; AGRAWAL, 2016). The intense urbanization and industrialization bring harmful micropollutants, which are not entirely eliminated during conventional water treatment and are not biodegraded by the environment either (BILA; DEZOTTI, 2007). In this way, the development of innovative tools to eliminate micropollutants presented in wastewaters is one of the main goals of environmental research in which the enzymatic route has been attracting attention (BA et al., 2013). Over the last years, enzymatic biocatalysts have been studied and developed to design a green and environmentally biocatalyst (VIRGEN-ORTÍZ et al., 2017; WOODLEY, 2008). The goal to design a green and environmentally biocatalyst to help to improve water quality emerges as one of the most relevant studies nowadays and laccase based biocatalysts have gained attention to achieve this purpose (DARONCH et al., 2020).

Laccase belongs to the group of phenol oxidases; it is a non-substrate specific enzyme and can catalyze the oxidation of phenolic substrates (RODRÍGUEZ COUTO; TOCA HERRERA, 2006). Their mechanisms of catalysis use four copper atoms present in their active site (DURÁN et al., 2002), and it has many applications such as textile and food industries, nanobiotechnology, cosmetics, removal of organic micropollutants from water, among others (BA et al., 2018; JORDAAN et al., 2009; MAYER; STAPLES, 2002; PEZZELLA; GUARINO; PISCITELLI, 2015). These characteristics make this enzyme suitable and attractive for industrial applications (UPADHYAY; SHRIVASTAVA; AGRAWAL, 2016). However, loss of stability, high cost of production, and non-reusability of soluble laccase can decrease its availability (BRUGNARI et al., 2018). In order to make these potential applications a reality, an improvement in laccase immobilization must be obtained (BRUGNARI et al., 2018).

Enzyme immobilization is an important challenge in biotechnology, and several methods can be employed to achieve stable, reusable, and resistance to different environmental factors biocatalysts (GUZIK; HUPERT-KOCUREK; WOJCIESZYŃSKA, 2014; RIOS et al., 2019). Enzyme immobilization on solid supports allows its easy recovery and reusability and improves the native enzyme properties, such as stability, activity, specificity, and selectivity (FERNANDEZ-LOPEZ et al., 2017). In order to achieve a well implemented immobilization, in conventional immobilization strategies, the selection of the support is an important step (BARBOSA et al., 2015; SANTOS et al., 2015b). For this,

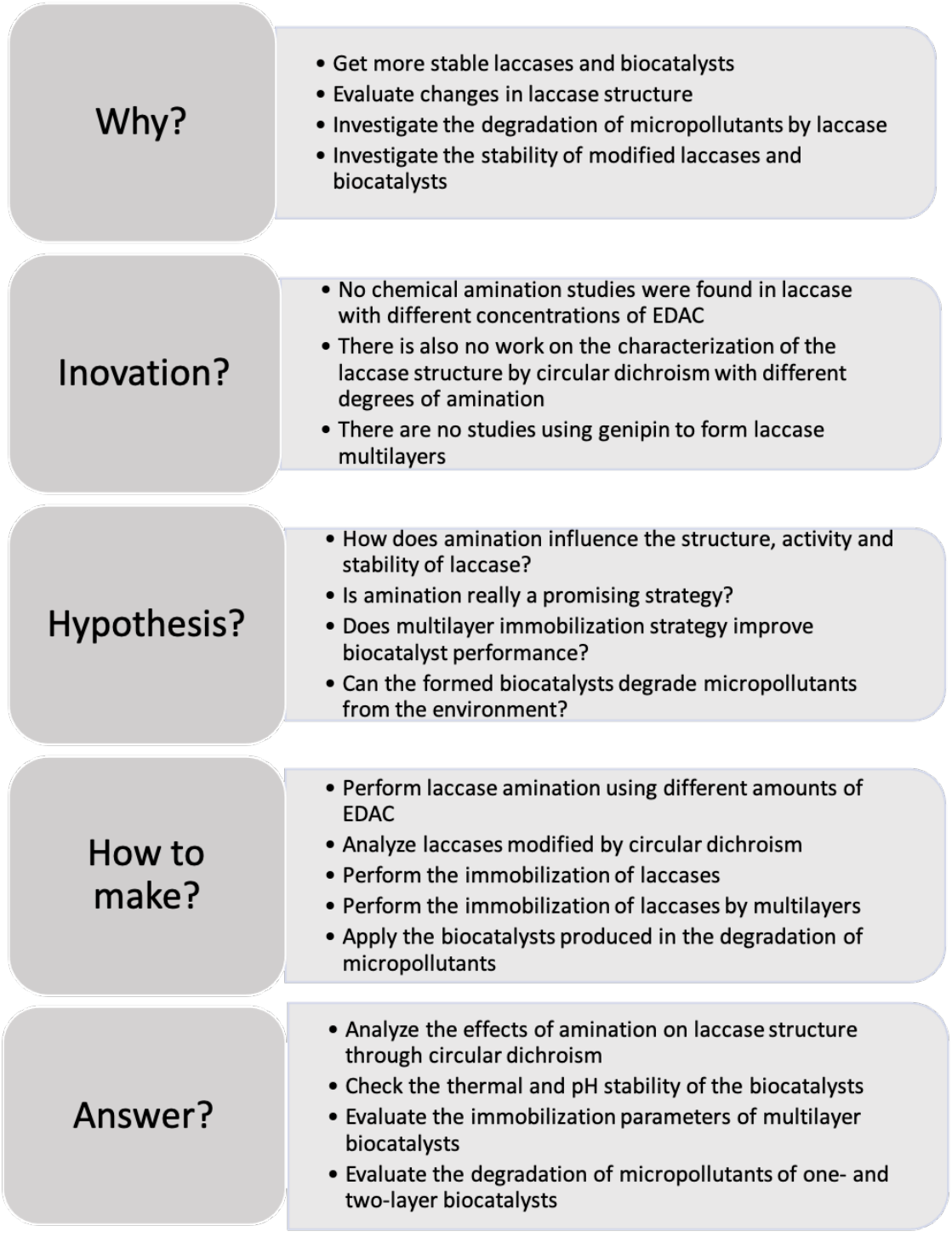
supports should be inert after enzyme immobilization and must have surfaces suitable for interaction with enzyme proteins and possess several reactive groups that can interact with them (CAO, 2005; CAO; LANGEN; SHELDON, 2003; HERNANDEZ; FERNANDEZ-LAFUENTE, 2011). In this context, agarose-based supports and chitosan were choose due to the surface area available to bind proteins, easy preparation, and biocompatibility (PESSELA et al., 2006; WAHBA, 2020).

The first approach of this study is the chemical amination of laccase following by ionic adsorption on cationic and anionic supports. Some enzymes from fungi, as laccases, presents low amounts of Lys residues (ADDORISIO et al., 2013), influencing the production of biocatalysts by covalent bonding. In this way, it comes to light the need for a chemical modification to improve the laccase properties. The chemical amination process allows the introduction of amino groups on the enzyme surface, which may enhance enzyme rigidity and stability (DE MORAIS JÚNIOR et al., 2017). In this process, the carboxylic group on the surface of the enzyme are activated by a water-soluble carbodiimide such as 1-ethyl-3-(3-dimethylamino propyl) carbodiimide (EDAC) (HOARE; KOSHLAND, 1966), then the activated carboxylic group forms an amide with one of the amino groups of the modifying reagent, such as ethylenediamine EDA (HOARE; KOSHLAND, 1967), as shown in Figure 3. This modification can be controlled by the concentration of EDAC (LÓPEZ-GALLEGO et al., 2005), and an increase in the concentration of EDAC in the enzymatic amination should allow the introduction of more amino groups into the enzyme. In this way, the immobilization by adsorption on charged supports such as diethyl aminoethyl-agarose (DEAE), monoaminoethyl-N-ethyl agarose (MANAE) (positively charged supports), and sulfopropyl (negatively charged supports), allows the observation of the increase of the enzyme amination.

The second approach of this study is the layer-by-layer immobilization of laccase onto chitosan activated with genipin or glutaraldehyde to enhance the loading capacity of the support (ARANA-PEÑA et al., 2020). In this context, chitosan was activated with genipin or glutaraldehyde in order to produce a heterofuncional support (BARBOSA et al., 2013), enabling the covalent immobilization of laccase. Afterward, the biocatalysts will be chemically coated with bifunctional agents (Glutaraldehyde – GA and Genipin – GEN), followed by the laccase immobilization step (second layer). The biocatalysts produced will be used in the degradation of acetaminophen (Tylenol, Dorfen, among others) and mefenamic acid; two drugs used for pain management that are not effectively removed by wastewater treatment processes being detected in low concentrations (ng/L) in treated effluents (CHEN et

al., 2016; MOLL et al., 2011; XU et al., 2018). In order to present the motivation of this study, a conceptual diagram of this work is shown in Figure 1.

Figure 1 – Diagram showing the motivations of this study.



1.2 Objective

The main objective of this project is to study protocols of immobilization of laccase (Novozym 51003) on different supports, producing highly active and stable biocatalysts to degradate micropollutants from water.

1.2.1 Specific objectives

- Study of the impact of EDAC quantities (0.024, 0.072 and 0.168 g) on laccase chemical amination;
- Characterization of laccase and aminated laccase by circular dichroism;
- Immobilization of laccase and aminated laccase on agarose-based supports with different charges (MANAE, DEAE, and sulfopropyl);
- Characterize the biocatalysts found with the purified and aminated enzyme relation with activity and stability (thermal and pH);
- Immobilization of laccase on chitosan activated with genipin or glutaraldehyde;
- Improvement of loading capacity of chitosan activated supports by immobilizing laccase by layer-by-layer technique;
- Characterize the biocatalysts produced by layer-by-layer technique in relation with activity and stability (thermal and operational);
- Degrade micropollutants from water: acetaminophen and mefenamic acid.

1.3 References

ADDORISIO, V.; SANNINO, F.; MATEO, C.; GUISÁN, J. M. Oxidation of phenyl compounds using strongly stable immobilized-stabilized laccase from Trametes versicolor. **Process Biochemistry**, [*s. l.*], v. 48, n. 8, p. 1174–1180, 2013.

ARANA-PEÑA, S.; RIOS, N. S.; MENDEZ-SANCHEZ, C.; LOKHA, Y.; GONÇALVES, L. R. B.; FERNÁNDEZ-LAFUENTE, R. Use of polyethylenimine to produce immobilized lipase multilayers biocatalysts with very high volumetric activity using octyl-agarose beads: Avoiding enzyme release during multilayer production. **Enzyme and Microbial Technology**, [*s. l.*], v. 137, p. 109535, 2020, 2019.

BA, S.; HAROUNE, L.; SOUMANO, L.; BELLENGER, J.; JONES, J. P.; CABANA, H. A hybrid bioreactor based on insolubilized tyrosinase and laccase catalysis and microfiltration membrane remove pharmaceuticals from wastewater. **Chemosphere**, [*s. l.*], v. 201, p. 749–755, 2018.

BA, S.; ARSENAULT, A.; HASSANI, T.; JONES, J. P.; CABANA, H. Laccase immobilization and insolubilization: from fundamentals to applications for the elimination of emerging contaminants in wastewater treatment. **Critical Reviews in Biotechnology**, [*s. l.*], v. 33, n. 4, p. 404–418, 2013.

BARBOSA, O.; TORRES, R.; ORTIZ, C.; BERENGUER-MURCIA, A.; RODRIGUES, R. C.; FERNÁNDEZ-LAFUENTE, R. **Heterofunctional supports in enzyme immobilization: From traditional immobilization protocols to opportunities in tuning enzyme properties**. [*s. l.*], 2013.

BECKER, D.; VARELA DELLA GIUSTINA, S.; RODRIGUEZ-MOZAZ, S.; SCHOEVAART, R.; BARCELÓ, D.; CAZES, M.; BELLEVILLE, M.; SANCHEZ-MARCANO, J.; GUNZBURG, J.; COUILLEROT, O.; VOLKER, J.; OEHLMANN, J.; WAGNER, M. Removal of antibiotics in wastewater by enzymatic treatment with fungal laccase – Degradation of compounds does not always eliminate toxicity. **Bioresource Technology**, [*s. l.*], v. 219, p. 500–509, 2016.

BILA, D. M.; DEZOTTI, M. **Desreguladores endócrinos no meio ambiente: Efeitos e conseqüências**. [*s. l.*], 2007.

BRUGNARI, T.; PEREIRA, M. G.; BUBNA, G. A.; FREITAS, E. N.; CONTATO, A. G.; CORRÊA, R. C. G.; CASTOLDI, R.; SOUZA, C. G. M.; POLIZELI, M. L. T. M.; BRACHT, A.; PERALTA, R. M. A highly reusable MANAE-agarose-immobilized Pleurotus ostreatus laccase for degradation of bisphenol A. **Science of the Total Environment**, [*s. l.*], v. 634, p. 1346–1351, 2018.

CAO, L. Immobilised enzymes: science or art? **Current Opinion in Chemical Biology**, [*s. l.*], v. 9, n. 2, p. 217–226, 2005.

CAO, L.; LANGEN, L.; SHELDON, R. A. Immobilised enzymes: carrier-bound or carrier-free? **Current Opinion in Biotechnology**, [*s. l.*], v. 14, n. 4, p. 387–394, 2003.

CHEN, C.; SUN, W.; LV, H.; LI, H.; WANG, Y.; WANG, P. Spacer arm-facilitated tethering of laccase on magnetic polydopamine nanoparticles for efficient biocatalytic water treatment. **Chemical Engineering Journal**, [*s. l.*], v. 350, n. 130, p. 949–959, 2018.

CHEN, P.; WANG, F. L.; YAO, K.; MA, J. S.; LI, F.; LV, W.; LIU, G. Photodegradation of mefenamic acid in aqueous media: Kinetics, toxicity and photolysis products. **Bulletin of Environmental Contamination and Toxicology**, [*s. l.*], v. 96, n. 2, p. 203–209, 2016.

DARONCH, N.; KELBERT, M.; PEREIRA, C. S.; ARAÚJO, P. H. H.; OLIVEIRA, D. Elucidating the choice for a precise matrix for laccase immobilization: A review. **Chemical Engineering Journal**, [*s. l.*], v. 397, p. 125506, 2020.

DE MORAIS JÚNIOR, W. G.; TERRASAN, C. R. F.; FERNÁNDEZ-LORENTE, G.; GUISÁN, J. M.; RIBEIRO, E. J.; RESENDE, M. M.; PESSELA, B. C. Solid-phase amination of Geotrichum candidum lipase: ionic immobilization, stabilization and fish oil hydrolysis for the production of Omega-3 polyunsaturated fatty acids. **European Food Research and Technology**, [*s. l.*], v. 243, n. 8, p. 1375–1384, 2017.

DURÁN, N.; ROSA, M. A.; D'ANNIBALE, A.; GIANFREDA, L. Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review. **Enzyme and Microbial Technology**, [*s. l.*], v. 31, n. 7, p. 907–931, 2002.

FERNANDEZ-LOPEZ, L.; PEDRERO, S. G.; LOPEZ-CARROBLES, N.; GORINES, B. C.; VIRGEN-ORTÍZ, J. J.; FERNANDEZ-LAFUENTE, R. Effect of protein load on stability of immobilized enzymes. **Enzyme and Microbial Technology**, [*s. l.*], v. 98, p. 18–25, 2017.

GUZIK, U.; HUPERT-KOCUREK, K.; WOJCIESZYŃSKA, D. Immobilization as a strategy for improving enzyme properties-application to oxidoreductases. **Molecules,** [*s. l.*], v. 19, n. 7, p. 8995–9018, 2014.

HERNANDEZ, K.; FERNANDEZ-LAFUENTE, R. Control of protein immobilization: Coupling immobilization and site-directed mutagenesis to improve biocatalyst or biosensor performance. **Enzyme and Microbial Technology**, [*s. l.*], v. 48, n. 2, p. 107–122, 2011.

HOARE, D. G.; KOSHLAND, D. E. A method for the quantitative modification and estimation of carboxylic acid groups in proteins. **The Journal of biological chemistry**, [*s. l.*], v. 242, n. 10, p. 2447–2453, 1967.

HOARE, D. G.; KOSHLAND, D. E. A procedure for the selective modification of carboxyl groups in proteins. **Journal of the American Chemical Society**, [*s. l.*], v. 88, n. 9, p. 2057–2058, 1966.

JORDAAN, J.; MATHYE, S.; SIMPSON, C.; BRADY, D. Improved chemical and physical stability of laccase after spherezyme immobilisation. **Enzyme and Microbial Technology**, [*s. l.*], v. 45, n. 6–7, p. 432–435, 2009.

LÓPEZ-GALLEGO, F. Improved stabilization of chemically aminated enzymes via multipoint covalent attachment on glyoxyl supports. **Journal of Biotechnology**, [*s. l.*], v. 116, n. 1, p. 1–10, 2005.

MAYER, A. M.; STAPLES, R. C. Laccase: new functions for an old enzyme. **Phytochemistry**, [*s. l.*], v. 60, n. 6, p. 551–565, 2002.

MOLL, R.; DERRY, S.; MOORE, R. A.; MCQUAY, H. J. Single dose oral mefenamic acid for acute postoperative pain in adults. **Cochrane Database of Systematic Reviews**, [*s. l.*], n. 3, 2011.

PESSELA, B.; FUENTES, M.; MATEO, C.; MUNILLA, R.; CARRASCOSA, A. V.; FERNANDEZ-LAFUENTE, R.; GUISAN, J. M. Purification and very strong reversible immobilization of large proteins on anionic exchangers by controlling the support and the immobilization conditions. **Enzyme and Microbial Technology**, [*s. l.*], v. 39, n. 4, p. 909–915, 2006.

PEZZELLA, C.; GUARINO, L.; PISCITELLI, A. How to enjoy laccases. **Cellular and molecular life sciences**, [*s. l.*], v. 72, n. 5, p. 923–940, 2015.

RIOS, N.; ARANA-PEÑA, S.; MENDEZ-SANCHEZ, C.; LOKHA, Y.; CORTES-CORBERAN, V.; GONÇALVES, L. R. B.; FERNANDEZ-LAFUENTE, R. Increasing the

enzyme loading capacity of porous supports by a layer-by-layer immobilization strategy using PEI as glue. **Catalysts**, [*s. l.*], v. 9, n. 7, p. 576, 2019.

RODRÍGUEZ COUTO, S.; TOCA HERRERA, J. L. **Industrial and biotechnological applications of laccases: A review**, [*s. l.*], 2006.

UPADHYAY, P.; SHRIVASTAVA, R.; AGRAWAL, P. K. Bioprospecting and biotechnological applications of fungal laccase. **3 Biotech**, [*s. l.*], v. 6, n. 1, p. 15, 2016.

VIRGEN-ORTÍZ, J. J.; SANTOS, J. C. S.; BERENGUER-MURCIA, Á.; BARBOSA, O.; RODRIGUES, R. C.; FERNANDEZ-LAFUENTE, R. **Polyethylenimine: A very useful ionic polymer in the design of immobilized enzyme biocatalysts**. [*s. l.*], 2017.

WAHBA, M. I. Enhancement of the mechanical properties of chitosan. **Journal of Biomaterials Science, Polymer Edition**, [*s. l.*], v. 31, n. 3, p. 350–375, 2020.

WOODLEY, J. M. New opportunities for biocatalysis: making pharmaceutical processes greener. **Trends in Biotechnology**, [*s. l.*], v. 26, n. 6, p. 321–327, 2008.

XU, B.; ZHAN, G.; XU, B.; DU, H.; LUO, H.; WANG, T.; ZHAN, C.; YANG, Y. Degradation of acetaminophen in aqueous solution by UV and UV-activated sludge processes. **Water Science and Technology**, [*s. l.*], v. 78, n. 10, p. 2088–2095, 2018.

Chapter 2

Literature review

2 LITERATURE REVIEW

In this literature review, the following topics will be addressed: oxidoreductases, laccase, laccase amination, immobilization, types of supports, immobilization methods and the application of free and immobilized laccase in the treatment of effluents.

2.1 Oxidoreductases

Oxidoreductases are enzymes involved in the biodegradation of lignin, phenol compounds, active pharmaceutical ingredients, flavors, fragrances, and other applications (MARTÍNEZ et al., 2017). They are classified as "green" catalysts, since they are most frequently employed in environmental applications involving the catalysis of the oxidation of various harmful and toxic chemicals (BILAL et al., 2017; ZDARTA et al., 2018). These enzymes present low substrate specificity and require substrates that show electron donor properties (NAGHDI et al., 2018b). Specifically, fungal oxidoreductases include 1) peroxidases and peroxygenases that are activated by H_2O_2 as sole electron acceptor; 2) oxidases containing flavin, which are activated by O_2 and other oxidants (such as Fe^{3+} and quinones); and 3) oxidases containing copper and that are activated by O_2, as the case of laccases (MARTÍNEZ et al., 2017).

Multi-copper oxidases belong to the copper-containing protein group and are ubiquitous in nature. These enzymes serve a very important role due to their participation in the electron transfer process in redox reactions, and in oxygen transport and activation (JANUSZ et al., 2020). Multicopper oxidases also reduce O_2 to H_2O using oxygen as an electron acceptor (JANUSZ et al., 2020). Moreover, their amino acid sequences contain a small cupredoxin-like domain and present a 3D structure that is relatively simple and primarily composed of beta-sheets and turns (JANUSZ et al., 2020). More complex processes involve the evolution of multicopper oxidases originating from different proteins, such as laccase.

2.1.1 Laccase general aspects

Laccases belong to the blue oxidase group (RODRÍGUEZ COUTO; TOCA HERRERA, 2006) and have been present in different organisms since their early evolutionary stages, making these proteins one of the earliest multicopper oxidases (JANUSZ et al., 2020).

Laccases can be found in a variety of fungi, bacteria, insects, and plants (YANG et al., 2017). Usually, these enzymes contain three domains; however, two-domain bacterial, mammalian, and coral laccases have also been discovered. Bacterial laccases are an important representative of monomeric intracellular proteins, presenting molecular weights in the range of 50-70 kDa and better performance in neutral to alkaline pHs (ARREGUI et al., 2019). These enzymes possess significant biochemical stability under various pH conditions, high temperatures, organic solvents, salt concentrations, and metal ions (ARREGUI et al., 2019; GUAN et al., 2018). Also, bacterial laccases are generally more robust and stable than their fungal counterparts and exhibit high tolerance to solvents such as ethanol, methanol, acetonitrile, and acetone. However, their applicability is restricted by their low redox potential of Type 1 copper (<+ 460 mV) compared with fungal laccases (GUAN et al., 2018; MATE; ALCALDE, 2015). The latter exist in a variety of structures and isoforms (i.e., multiple enzyme forms with the same substrate specificity) (ANAH IACUTE et al., 2016), which is due to different factors, including culture conditions, fungus species, and the specific strain used (ANAH IACUTE et al., 2016). Additionally, these glycoproteins have molecular weights in the range of 60 to 70 kDa and an isoelectric point around pH 4.0 (BALDRIAN, 2006). The glycosylation range of these enzymes generally increase 10–25% in mass (although some laccases present a >30% increase), with the presence of some N-linked carbohydrates (BERKA et al., 1997). These carbohydrate portions are responsible for ensuring their conformational stability, and protection against proteolysis and inactivation by radicals (ARREGUI et al., 2019).

The biological importance of laccase is attributed to its large substrate oxidation capacity (ERNST et al., 2018; GUAN et al., 2018). The enzyme presents four copper atoms in their catalytic sites, which are classified into three types, and distributed in different connections (DURÁN et al., 2002). Laccases catalyze the oxidation of aromatic mono-, di- and polyphenols, aminophenols, methoxy phenols, and aromatic amines with the reduction of oxygen in water to form less harmful pollutants (ARREGUI et al., 2019; DURÁN et al., 2002). These highly numerous organic and inorganic substrates grant laccases a variety of catalytic and protective functions against adverse environment conditions (JANUSZ et al., 2020). During catalysis, substrates are required to present a heterocyclic or aromatic ring, easily oxidized substitutions (hydroxyl groups), and electron donor substituents (phenyl, alkyl, among other groups) (NAGHDI et al., 2018b; TRAN; URASE; KUSAKABE, 2010). Another advantage is that laccases only require oxygen in gas form as a co-substrate, which makes it even more technologically interesting (NAGHDI et al., 2018b; NGUYEN, L. N. et

al., 2014). Therefore, due to their low catalytic requirements and good ability to catalyze degradation or polymerization reactions, they have been applied in a wide variety of industrial processes (MORENO et al., 2020). To this end, however, there is still a need for better enzyme stability, water solubility, and higher inhibition properties (against substrates, products, or non-related compounds) (VIRGEN-ORTÍZ et al., 2017a). As such, to render these potential applications feasible, laccase low solubility and the high costs associated with these biocatalysts should be addressed. In this context, the development and optimization of the catalytic activity of laccases have been studied by different engineering approaches such as immobilization, directed evolution, and computational methods (MORENO et al., 2020).

2.1.1.1 Novozym 51003

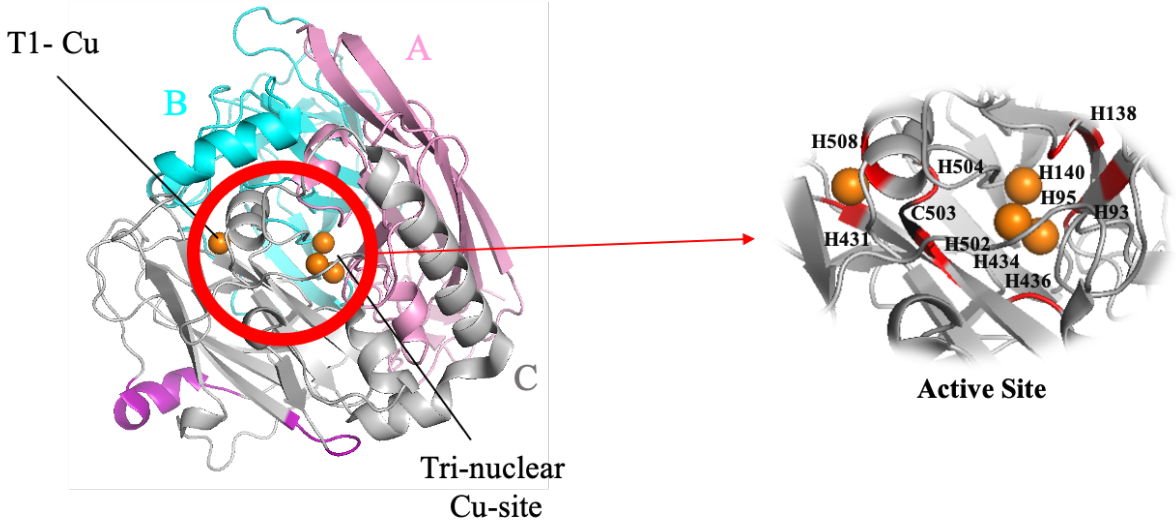
Novozym 51003 is an extracellular laccase from *Myceliophthora thermophila* expressed in *Aspergillus oryzae* (KALYANI et al., 2012; PINAR et al., 2017; PLAGEMANN; VON LANGERMANN; KRAGL, 2014). This thermophilic fungus can produce enzymes with pronounced thermal resistance, and the Aspergillus species provide expression systems for the production of industrial enzymes (BERKA et al., 1997). Novozym 51003 presents a pI of 4.2, a molecular weight between 75 and 95 kDa (measured by SDS-PAGE), and a 33 to 60% level of glycosylation, of which 14% involve N-linked carbohydrates. According to literature (BERKA et al., 1997), the purified enzyme has 23 mol of glucosamine, 38 mol of galactose, 4 mol of glucose, and 85 mol of mannose per mol of the enzyme. Literature data also suggest that these enzymes present isoforms with no significant differences, as confirmed by SDS-PAGE.

2.2 Active site and catalytic cycle

The mechanism of action of laccases involves the formation of radical species, which can result in dimers or polymers generated by oxidative coupling reactions, or the production of dead-end products via intramolecular rearrangements (BASSANINI et al., 2020). Depending on the organism from which they originate, laccases may contain two or three cupredoxin-like domains, which bind copper (Cu) centers involved in the inter-molecular electron transfer reactions and constitute the catalytic site (BASSANINI et al., 2020). The active site of laccases contains four Cu atoms classified, according to Electron Paramagnetic Resonance (EPR), into three types: Type 1 (blue site), Type 2 (normal site), or

Type 3 (binuclear site, which consists of two antiferromagnetically copper atoms coupled) (SUNDARAM et al., 1997). The Type 1 copper atom (T1 – Cu) has strong electronic absorption and is EPR detectable (FERNÁNDEZ-FERNÁNDEZ; SANROMÁN; MOLDES, 2013). It is coordinated by two histidines and one cysteine residue and is responsible for electron capture (ERNST et al., 2018). Type 2 copper (T2 – Cu) is also still EPR detectable, even if it is colorless (FERNÁNDEZ-FERNÁNDEZ; SANROMÁN; MOLDES, 2013). Two histidine along with water molecules coordinate the center of copper type 2 as a binder. Type 3 copper is not EPR detectable and consists of two copper atoms with weak absorbance near the standard UV spectrum (FERNÁNDEZ-FERNÁNDEZ; SANROMÁN; MOLDES, 2013). Type 2 and 3 coppers form a trinuclear structure coordinated by histidine residues that promote oxygen reduction in water (DURÁN et al., 2002). A scheme of the catalytic site of laccase is shown in Figure 2.

Figure 2 - Schematic representation of the Novozym 51003 structure. The four catalytic Cu ions are shown as orange spheres, including ten histidine residues (in red) and one cysteine residue (in black) that act as Cu-ligands. The three cupredoxin-like domains are colored as follows: domain A (1-161) *pink*, domain B (162-341) *light blue*, and domain C (342-559) *grey*. ABTS binding sites (in purple) are presented in the domain C of laccase (loops 410-414, 460-469, and 347-351). The structure was taken from the Protein Data Bank (PDB) using PyMOL Educational, PDB code: 6F5K



Source: elaborated by the author.

The catalytic activity of laccases occurs by electron transfer and involves the reduction of O_2 and oxidation of substrates (GAMALLO et al., 2018). It initiates with the

oxidation of four substrates molecules to form four organic radicals, while one molecule of oxygen is reduced to form two water molecules (BASSANINI et al., 2020; KALYANI et al., 2012). The Type 1 copper captures the electron from the substrate and it is subsequently reduced from Cu (II) to Cu (I) (XU, F., 1997). This is followed by an internal electron transfer from the Type 1 copper to the Type 2 and 3 copper centers (GAMALLO et al., 2018), simultaneously to the oxidation of the substrate and the reduction of O_2 to H_2O at these centers (GIANFREDA; XU; BOLLAG, 1999). The rate-limiting step of the laccase catalytic mechanism is the reduction of the Type 1 copper. Apart from this, the laccase substrate array is limited to molecules containing phenolic moieties due to the low potential values of Type 1 copper redox (from 420 to 790 mV) (GIARDINA et al., 2010; JANUSZ et al., 2020). The redox potential of laccases is related to the energy required to remove an electron from the reducing substrate (ARREGUI et al., 2019). Accordingly, laccases are divided into low-redox-potential laccases (derived from bacteria, plants, and insects) and high-redox-potential laccases (widely present in fungi) (MUNK et al., 2015).

Laccases catalyze the oxidation of a wide range of substrates. However, in most cases, their substrates present poor solubility in water. Their oxidation efficiency depends on the difference between the redox potentials of the substrate and the Type 1 copper (YANG et al., 2017). Besides, the elements of steric hindrance, active site penetration, and redox potential incompatibility can hinder the direct oxidation of some substrates (BASSANINI et al., 2020). In such scenarios, it is necessary that a mediator compound act as an intermediate substrate allowing laccase to indirectly oxidize substrates of higher redox potential. The most commonly-used synthetic mediators are 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) and 1-hydroxy benzotriazole (HBT), while natural phenolic mediators include syringaldehyde (SA) and acetosyringone (AS) (YANG et al., 2017).

These classes of mediators have different catalytic mechanisms. In the case of ABTS-mediated substrates, an electron transfer occurs along with the oxidation of ABTS to its radical cation ($ABTS^{\cdot+}$, 472 mV), and then to the di-cation ($ABTS^{2+}$, 885 mV) (YANG et al., 2017). HBT-mediated substrates (N-OH type mediator), on the other hand, form the N-oxy radical after laccase oxidation, which abstracts the benzylic hydrogen atom from the substrate (YANG et al., 2017). Phenolic-mediated substrates follow a radical hydrogen abstraction mechanism with a phenoxy radical as intermediate (YANG et al., 2017).

ABTS single-electron oxidation is the most frequently employed reaction model of laccase activity due to the easily detectable color change following $ABTS^{2+}$ formation (ITOH; TAKAGI, 2021). The binding site described for ABTS (loops 410-414, 460-469, and

347-351) is presented in the domain C of laccase (Figure 2) (ERNST et al., 2018). The literature reports a decrease in the oxidation rate of ABTS with the increase of pH (SONG et al., 2021). Also, previous works reported that some compounds such as sodium chloride, methanol, and ethanol (CHAMPAGNE; NESHEIM; RAMSAY, 2013; MALVIYA et al., 2014; RE et al., 1999) could inhibit ABTS oxidation. These results are in accordance with the literature, since anions such as CN^-, F^-, Cl^- and OH^- can indeed inhibit laccase activity by binding to the Type 2/Type 3 coppers and interrupting the internal electron transfer from copper Type 1 to Type 2/Type 3 center (LIU, Y. et al., 2017).

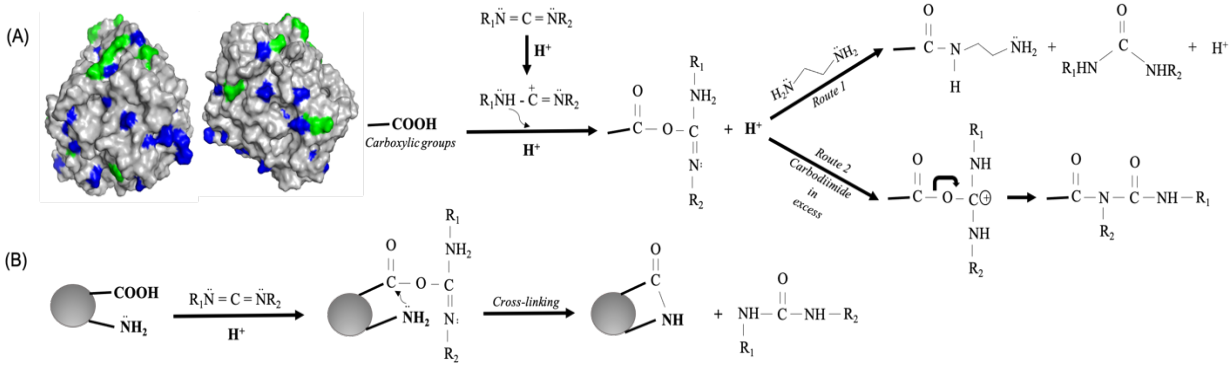
2.3 Chemical amination of laccase

In general, enzymes from fungi, such as laccases, present low amounts of Lys residues (ADDORISIO et al., 2013), which are widely used due to the important role they play in the interaction between enzyme and support. These residues are usually placed on the molecule surface due to their hydrophilicity, and the amino group of lysine is a nucleophilic molecule that allows its direct reaction with the support (RODRIGUES, R. C. et al., 2014). Another key observation to be made is that the isoelectric point of laccase is at pHs 4 to 5 (ERNST et al., 2018; RODRIGUES, R. C. et al., 2014), indicating the prevalence of negative charges on its surface. Thus being, to solve this issue, a chemical amination can be performed to improve enzyme features and modulate its properties. Chemical amination introduces primary amino groups (NH_2^+) onto the enzyme surface, leading to an alteration of the isoelectric point of the protein and its chemical reactivity, as well as an increase in the enzyme rigidity and stability (RODRIGUES, R. C. et al., 2014). However, the effect on the enzyme features may be hard to predict, since alterations in the ionic interactions at the protein surface may occur due to ionic bridges being broken and changed by repulsion forces (RODRIGUES, R. C. et al., 2014). This process may then alter the existing interactions between the groups on the enzyme surface and tune its properties affecting its conformation, stability, activity, specificity, and/or selectivity (RODRIGUES, R. C. et al., 2014).

The most common strategy for chemical amination involves the activation of the carboxyl group of a protein by a water-soluble carbodiimide, followed by the reaction of the activated carboxyl groups with a nucleophile (FERNANDEZ-LORENTE et al., 2008; HOARE; KOSHLAND, 1966, 1967; HOARE; OLSON; KOSHLAND, 1968; LÓPEZ-GALLEGO et al., 2005a; MATYASH; OGLOBLINA; STEPANOV, 1973; PERFETTI; ANDERSON; HALL, 1976). However, these carbodiimides and nucleophiles can further

react with other reactive groups of proteins such as sulfhydryl and phenolic groups (CARRAWAY; KOSHLAND, 1968; CARRAWAY; TRIPLETT, 1970). Consequently, the chemical amination process increases the amount of reactive groups by modifying these groups by a variety of reagents used to this end (HOARE; KOSHLAND, 1967). When ethylenediamine is used as the nucleophile, for example, the introduced amino groups will be more reactive than the Lys group because the former present a lower pKa value than the latter (RODRIGUES, R. C. et al., 2014). A schematic diagram, showing the amination process in the different residues of Novozym 51003, is presented in Figure 3 and 4.

Figure 3 - (A) Reactions between carbodiimide and carboxyl groups of Novozym 51003; glutamic residues are represented in green and aspartic residues, in blue. (B) Reactions between carbodiimide and sulfhydryl groups of cysteine (in orange) in Novozym 51003. The structure was taken from the Protein Data Bank (PDB) using PyMOL Educational, PDB code: 6F5K



Source: elaborated by the author.

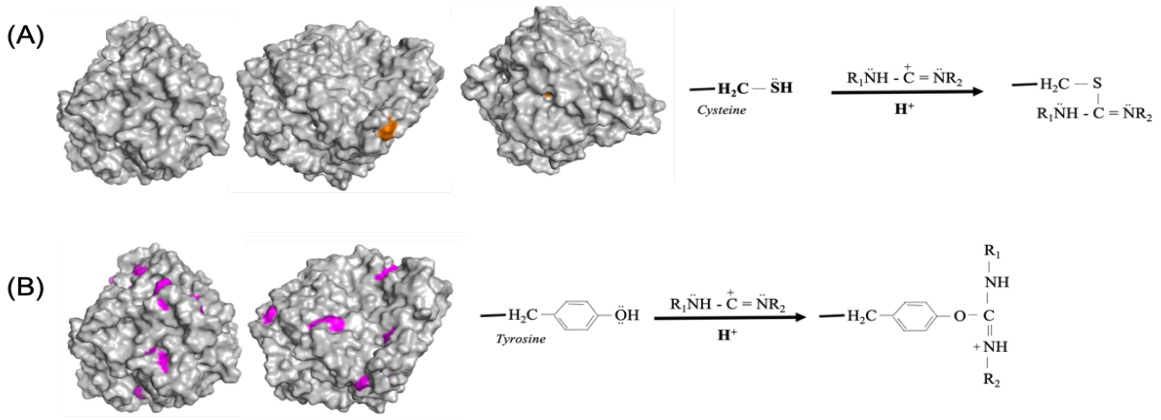
Controlling the concentration of EDAC in the medium allows the control of the percentage of modified carboxyl groups (LÓPEZ-GALLEGO et al., 2005a). Thus being, the mechanism involving the activation of the carboxyl group with a water-soluble carbodiimide, such as EDAC, can take two routes, as described in Figure 3A. For this reaction to take place, the pH must be at 4.75 for the dissociation of the carboxylic groups, since ionized carboxyl groups are required to react with the carbodiimide (NAKAJIMA; IKADA, 1995). In the first route, the mechanism involves the formation of an O-acyl-iso-urea intermediate due to the activation of the hydroxyls present in the carboxyl groups (terminal carboxylic, glutamic, and aspartic acids) by the carbodiimide (HOARE; KOSHLAND, 1966, 1967; NAKAJIMA; IKADA, 1995). This intermediate is subsequently changed into a carbocation due to the re-

protonation at the site of the Schiff's base (RODRIGUES, R. C. et al., 2014). Finally, the reaction with the amino groups of a nucleophile, such as ethylenediamine (EDA), takes place to form an amide (Figure 3A, route 1) (HOARE; KOSHLAND, 1967).

In the second route, the intermediate O-acyl-iso-urea forms N-acyl-urea via an intramolecular acyl transfer (Figure 3A, route 2). In this mechanism, if the nucleophile concentration is high, the rearrangement is slow compared to the nucleophilic attack mechanism (RODRIGUES, R. C. et al., 2014). Also, the carbodiimide concentration influences both mechanisms, since in lower concentrations of carbodiimide, route 1 takes place, while in solutions with a higher concentration of carbodiimide, route 2 is followed (HOARE; KOSHLAND, 1967; NAKAJIMA; IKADA, 1995). A third mechanism can also occur, triggering an intramolecular cross-linking between neighboring nucleophiles with enzyme inhibition (Figure 3B) (RODRIGUES, R. C. et al., 2014).

Carbodiimides can also react with free sulfhydryl groups of proteins, as shown in Figure 4A. At acidic pHs, a higher concentration of carbodiimide can almost completely modify sulfhydryl groups (CARRAWAY; TRIPLETT, 1970). This reaction has highly similar reaction rates to those of carboxyl groups, but in proteins, there are expected variations regarding modification rates due to protein structure (CARRAWAY; TRIPLETT, 1970). Although carbodiimide has a strong preference for carboxyl groups, reactions with phenolic groups have also been detected (Figure 4B) (CARRAWAY; KOSHLAND, 1968). The rate of this reaction is slow compared to those of carboxyl groups and it generates O-aryl isourea as a product (RODRIGUES, R. C. et al., 2014).

Figure 4 - (A) Reactions between carbodiimide and phenolic groups of tyrosine (in purple) in Novozym 51003. (B) Intramolecular cross-linking. The structure was taken from the Protein Data Bank (PDB) using PyMOL Educational, PDB code: 6F5K



Source: elaborated by the author.

2.4 Immobilization methods

High enzymatic activity and good ability to degrade large amounts of phenolic compounds render laccases highly suitable for different industrial sectors (FERNÁNDEZ-FERNÁNDEZ; SANROMÁN; MOLDES, 2013; MATEO, CESAR et al., 2007; SU et al., 2018). However, practical applications of laccase require the preparation of stable and reusable derivatives (ZHANG, X. et al., 2015). Therefore, the immobilization of laccase appears as a pertinent method for improving its properties, especially thermal stability and resistance to harsh conditions and to chemical reagents (FERNÁNDEZ-FERNÁNDEZ; SANROMÁN; MOLDES, 2013). In general, enzyme immobilization is a critical step in the preparation of most biocatalysts (PINHEIRO et al., 2019) and, in order to achieve a well-implemented enzyme immobilization protocol, a few important elements should be considered, such as methods, support characteristics, and the use of crosslinkers.

Enzyme immobilization is not only a key step to solve hurdles concerning enzyme reusability, but it is also a powerful tool to improve native enzyme properties, such as stability, activity, specificity, and selectivity (FERNANDEZ-LOPEZ et al., 2017). In this way, the several methods employed to achieve well-established enzyme immobilizations can be classified into physical and chemical methods. Examples of physical methods involve adsorption, entrapment, and encapsulation, while chemical methods involve cross-linking and covalent bonding (RAFIEE; REZAEE, 2021). Methods of enzyme immobilization can also be classified based on their reversibility. Reversible methods encompass protocols in which the enzyme can be separated from the support without destroying the biological activity of the enzyme or the support, while irreversible methods are the ones in which the separation will lead to the destruction of the enzyme's biological activity (RAFIEE; REZAEE, 2021). In the following sections, some of these methods, vastly used for laccase immobilization, are discussed in detail.

2.4.1 Adsorption

Among the vast array of immobilization techniques, adsorption is the simplest of them, and it involves a binding of the enzyme to the support via interactions of ionic (van der Waals forces, hydrogen bonding) or hydrophobic nature (REIS et al., 2019). The technique is simple, rapid, and follows a very flexible protocol, which results in it being one of the most used techniques (FUENTES et al., 2004; JESIONOWSKI; ZDARTA; KRAJEWSKA, 2014;

REIS et al., 2019). It is also an economical method, since it involves high enzyme activities, low costs regarding carrier materials and immobilization procedures, no need for chemical additives, and the possibility of support regeneration (RAFIEE; REZAEE, 2021).

The main drawback of the method is the common leakage of enzymes due to the inherently weak interactions with the support (RAFIEE; REZAEE, 2021). However, some enzyme immobilizations on hydrophobic supports can provide stronger interactions, which can circumvent the issue (RAFIEE; REZAEE, 2021). This is the case of laccase, which immobilization through hydrophobic interactions is favored since there are more aromatic residues exposed in its surface (LAI et al., 2013). Enzyme properties (such as isoelectric points, surface characteristics) and medium characteristics (e.g., pH, ionic strength, and enzyme/support ratio) are reported to influence the efficiency and robustness of the immobilization technique (PESSELA et al., 2004; RAFIEE; REZAEE, 2021). In general, this specific protocol provokes small conformational changes to the enzyme due to the rather weak binding, except in the case of lipases (JESIONOWSKI; ZDARTA; KRAJEWSKA, 2014; OVERBEEKE et al., 2000), and enables the support recovery after protein inactivation, once the enzyme can be desorbed from it at the end of the cycle (MATEO, CESAR et al., 2000).

The physical adsorption interactions between enzyme and support will determine the potential biocatalyst applications, given the fact that better adsorption is obtained when more robust interactions occur (SANTOS et al., 2015). Thus being, the support structure should be able to show multi-interaction properties to immobilize different proteins (GARCIA-GALAN et al., 2011). The adsorption of proteins on ionic exchangers, for example, requires multipoint adsorption, where several groups in the support interact with several groups in the proteins, increasing adsorption strength (PESSELA et al., 2006). In this way, the main enzyme areas involved in multipoint processes are those possessing higher concentrations of the target groups (BARBOSA et al., 2013). Additionally, support porosity plays an important role in the amount of adsorbed enzymes and their catalytic activity (RAFIEE; REZAEE, 2021). This is because the size and distribution of the pores influence the properties of immobilized enzymes: in supports of small pore sizes, problems with diffusion limitation resulting in structural rearrangement of the enzymes with subsequent inactivity can occur, while for large-pore supports, issues with enzyme clusters and a consequent activity decrease can be observed (MILETIĆ; NASTASOVIĆ; LOOS, 2012; RAFIEE; REZAEE, 2021). Also, the surface area changes with pores sizes and narrower pore

diameters restrict the area effectively available for enzyme immobilization (BAYNE et al., 2013).

2.4.2 Immobilization by covalent attachment

This technique is one of the most widely used enzyme immobilization methods, but it can be more complex to achieve, since some supports require some form of preliminary activation (GARCIA-GALAN et al., 2011). Also, there are some important factors that can influence the immobilization process, such as the composition, size, and shape of the support material, the nature of the coupling method, the conditions during coupling, and the direction of the enzyme binding to the support (RAFIEE; REZAEE, 2021). Initially, the enzymes' functional groups are adsorbed and then covalently linked to the support, which leads to an increase in their stability and inhibition of their leaching into solutions (RAFIEE; REZAEE, 2021). In this process, only the non-ionized amino groups act as good nucleophiles and are part of the enzyme immobilization (HERNANDEZ; FERNANDEZ-LAFUENTE, 2011). In general, the functional groups involved, such as side chains of lysine (ε-amino group), cysteine, aspartic, and glutamic residues, as well as imidazole and phenolic groups (MOHAMAD et al., 2015), are not essential for their catalytic activity. Biocatalysts with the highest activities are generated when the active center is not involved in the binding process to the support (MOHAMAD et al., 2015).

Multipoint covalent bonding occurs when the enzyme is attached to the support by multiple covalent chains (RAFIEE; REZAEE, 2021). The groups involved with immobilizations by multipoint covalent attachment cannot alter their relative positions by distances longer than the size of the spacer arm (SANTOS et al., 2015). This makes this specific technique a powerful tool to improve enzyme rigidity and consequently, enzyme stability (SANTOS et al., 2015). If a very intense multipoint covalent attachment is achieved, the enzyme structure will be fixed and rigid, which protects the enzyme from denaturation against heat, organic solvents, and extreme pH conditions (RAFIEE; REZAEE, 2021). Another point to be considered is that this technique should prevent enzyme conformational changes that are induced by any reagent, which also helps to maintain enzyme activity under acceptable conditions (RODRIGUES, R. C. et al., 2013). To this purpose, the support matrix should be rigid and the spacer arm, short, allowing the enzyme groups involved in the immobilization to maintain their relative positions, since one group cannot move regardless of others (RODRIGUES, R. C. et al., 2013). Thus being, the only means of altering enzyme orientation on the support is by altering the step that promotes the first immobilization

(HERNANDEZ; FERNANDEZ-LAFUENTE, 2011). Multipoint covalent bonding of enzymes may not be simple to achieve, but it is one of the most powerful strategies aiming at enzyme stabilization (RODRIGUES, R. C. et al., 2013).

2.4.3 Layer-by-layer immobilization

Layer-by-layer immobilization is a method that occurs by placing multiple enzyme layers on top of each other, thus forming enzyme multilayers (ARANA-PEÑA; RIOS; MENDEZ-SANCHEZ; LOKHA; GONÇALVES; et al., 2020; FERNÁNDEZ-FERNÁNDEZ; SANROMÁN; MOLDES, 2013). Typically, the layers are built by alternating layers of enzyme and materials bearing opposite charges, until the desired number of layers is achieved (NGUYEN, H. H.; KIM, 2017). However, as electrostatic interactions require charged layers, other possibilities of building layer-by-layer biocatalysts were explored (ZHANG, J. et al., 2020). Therefore, multilayer biocatalysts can be formed by interactions such as electrostatic interactions, hydrogen bonding, covalent bonding, hydrophobic interactions, or by a combination of these (NGUYEN, H. H.; KIM, 2017; ZHANG, J. et al., 2020). This immobilization technique has some advantages, such as the diversity of natural and artificial materials, stability, and controllability (ZHANG, J. et al., 2020). Also, this strategy is proposed for adding a higher concentration of the desired enzyme so as to enhance the loading capacity of the support and increase the overall activity of the biocatalyst (ARANA-PEÑA; RIOS; MENDEZ-SANCHEZ; LOKHA; GONÇALVES; et al., 2020). However, overcharging the enzyme surface may cause partitioning or diffusion phenomena that can compromise its stability (NGUYEN, H. H.; KIM, 2017).

In general, this method has been mainly used with non-porous supports, but it can also be used to improve the immobilization of enzymes on porous materials (ARANA-PEÑA; RIOS; MENDEZ-SANCHEZ; LOKHA; GONÇALVES; et al., 2020; ZHANG, J. et al., 2020). In the latter scenario, each enzyme layer added generates a decrease in the pore radius, which renders the stacking of several enzyme layers more complex to achieve (RIOS et al., 2019). In this way, diffusional problems in the enzyme and substrate are expected once the support pore radius have been decreased (ARANA-PEÑA; RIOS; MENDEZ-SANCHEZ; LOKHA; GONÇALVES; et al., 2020). Using non-porous supports, the substrate diffusion from the upper enzyme layers to the lower enzyme layer (the one in direct contact with the support) is the only relevant diffusion-related problem (RIOS et al., 2019).

2.5 Supports for immobilization

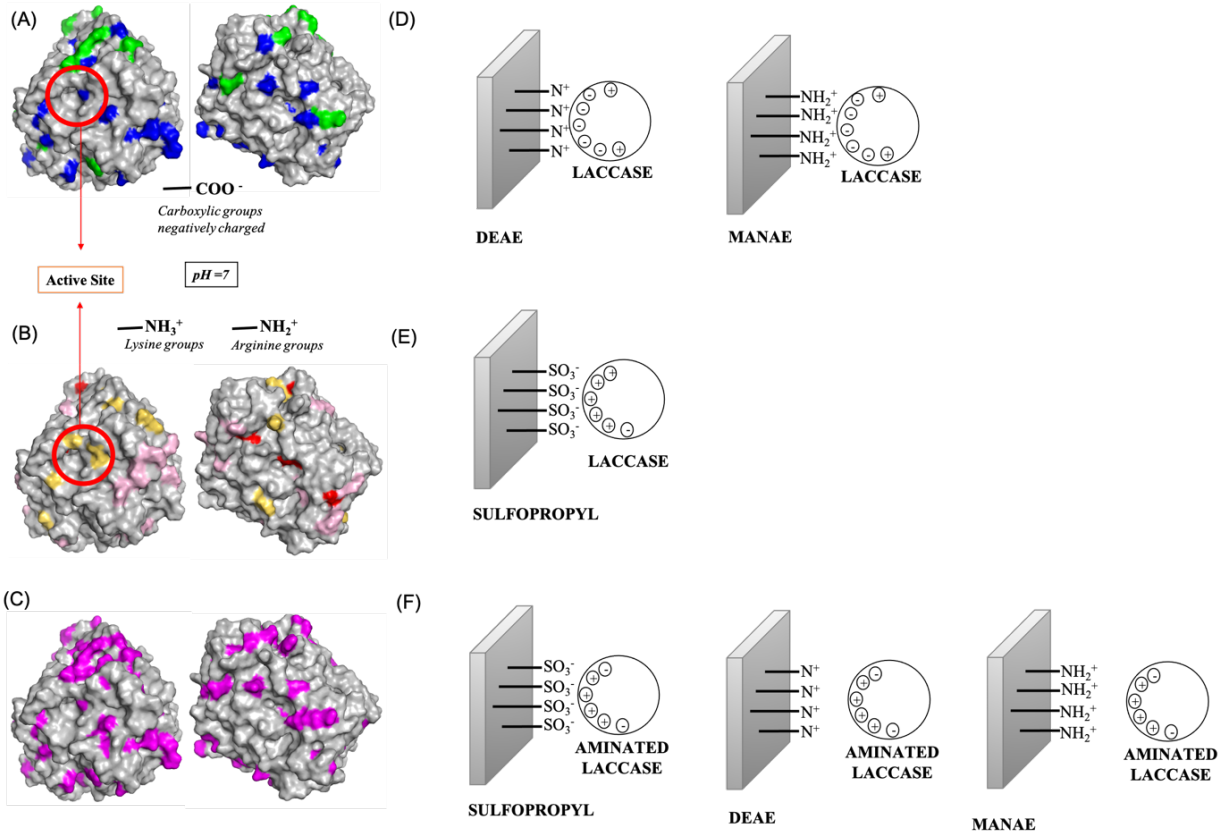
Enzyme immobilization is an important tool for facilitating the reuse of biocatalysts and improving enzyme properties of stability, selectivity, specificity, purity, among others (RIOS et al., 2019). In conventional immobilization strategies, adequate support selection is paramount for achieving a successful immobilization (RIOS et al., 2019). For this, supports must have surfaces that are suitable for interaction with enzyme proteins and possess several reactive groups that can interact with them (CAO, 2005; CAO; LANGEN; SHELDON, 2003). Also, ideal supports should be inert after enzyme immobilization has taken place so as to avoid enzyme inactivation due to undesired interactions between the support and the immobilized proteins, during their storage or application (HERNANDEZ; FERNANDEZ-LAFUENTE, 2011). Besides, costs are also relevant, but can be lowered, depending on the enzyme loading capacity and the process improvements after their respective immobilization protocols (RIOS et al., 2019). In the following sections, some supports commonly used for enzyme immobilization are discussed in detail.

2.5.1 Ion exchange supports

One of the most used reversible immobilization techniques is the ionic adsorption of enzymes onto ionic exchange resins (FILHO et al., 2008), due to them being very rapid and simple (PESSELA et al., 2006). In ion exchange, different regions of the enzyme can be involved in the immobilization process depending on the experimental conditions and the activation degree of the support (BARBOSA et al., 2013). A higher number of enzyme-support interactions will be required to bind an enzyme onto these supports the higher the ionic strength of this support (BARBOSA et al., 2015). Thus, the adsorption strength is related to the number of enzyme groups interacting with the support groups (PESSELA et al., 2006). The environmental conditions under which ion exchange immobilizations are performed are of fundamental importance to the replacement of the support ions by the ionized groups of the enzyme (HERNANDEZ; FERNANDEZ-LAFUENTE, 2011). This means that the pH and ionic strength of the medium must be controlled to achieve good immobilization and process efficiency (JÚNIOR, 2017). However, the main drawback of this protocol is the high degree of enzyme leaching observed during the use of biocatalysts due to changes in pH or ionic strength, which leads to product contamination and biocatalyst inactivation (PESSELA et al., 2006).

In weakly activated supports, large proteins become adsorbed much more easily than small proteins, while highly activated ionic exchangers can strongly adsorb both large and small protein units (PESSELA et al., 2006). In this way, large surfaces of highly activated supports make them the best option to promote protein interaction (PESSELA et al., 2006). Agarose, for example, offers a large surface for protein interaction, enabling a very intense adsorption of large proteins (PESSELA et al., 2006). In this context, monoaminoethyl–N–aminoethyl (MANAE) is an anionic support obtained from the modification of epoxy groups of agaroses with ethylenediamine (EDA). The adsorbent properties on this support can be altered by changing the pH value since they present both double positive charges at neutral or acid pHs, and one charge at moderately alkaline pHs (PESSELA et al., 2006). Another anionic support is diethylamino ethyl-agarose (DEAE), which is a commercially available ion exchange support activated with very strong ionic groups, and that can provide strong enzyme-support interactions (PESSELA et al., 2004). Sulfopropyl is another commercial support, and it presents the functional groups $CH_2CH_2CH_2SO_3^-$ exposed in its surface (cationic support). These anionic and cationic supports have ionizable functional groups that react specifically with enzyme functional groups (JÚNIOR, 2017). As such, different enzyme orientations after immobilization can be achieved using these supports, since anionic supports can interact with negative residues of enzymes, while cationic supports can interact with their positive counterparts. In this work, Novozym 51003 and aminated Novozym 51003 were immobilized in different ionic exchange supports and a schematic representation of the interaction of the exposed residues of each laccase with each support are presented in Figure 5.

Figure 5 - (A) Negative residues in laccase structure: glutamic residues (in green) and aspartic residues (in blue). (B) Positive residues in the laccase structure: histidine residues (in red), arginine residues (in pink), and lysin residues (light yellow). (C) Exposed residues in laccase structure capable of amination (glutamic, aspartic, tyrosine, and cysteine). (D) Non-modified laccase adsorption onto DEAE-agarose and MANAE-agarose (anionic supports). (E) Non-modified laccase adsorption onto sulfopropyl (cationic support). (F) Modified laccase adsorption onto sulfopropyl (cationic support) and DEAE-agarose and MANAE-agarose (anionic supports). The structure was taken from the Protein Data Bank (PDB) using PyMOL Educational. PDB code: 6F5K



Source: elaborated by the author.

As presented in Figure 5, the negative residues of laccase (Figure 5A) should interact with the anionic supports, such as MANAE and DEAEA-agarose, to form multiple interactions (Figure 5D). On the other hand, laccase should be immobilized in sulfopropyl (Figure 5E) by the positive residues present on its surface (Figure 5B). The amination process introduces amine groups (NH_2^+) onto the laccase surface (Figure 5C), leading to an increase in the number of interactions with the cationic support (sulfopropyl) instead of anionic supports (Figure 5F). As previously mentioned, the concentration of EDAC in the medium

allows the controlling of the percentage of modified carboxyl groups (LÓPEZ-GALLEGO et al., 2005a). This way, as more EDAC is added during the process, it is expected that a predominance of positive groups on the laccase surface takes place.

Support hydrophobicity/hydrophilicity is a physical property that also affects enzyme-support interactions (BARBOSA et al., 2015). For example, the hydrophilicity of chitosan, due to the amino and hydroxyl groups in its structure, can provide additional stability to the enzyme, preserving its active conformation under high organic solvent concentrations, once this support can ensure the retention of the necessary water molecules in the enzyme molecular microenvironment (BARBOSA et al., 2015; DU et al., 2020; ZAAK et al., 2017). Another example is the immobilization of lipases on hydrophobic supports at low ionic strengths, which allows lipase immobilization and stabilization under its open form (BARBOSA et al., 2015; ZAAK et al., 2017). In the case of laccase, when it is in its active form, immobilization through hydrophobic interactions is favored since there are more aromatic residues exposed in its surface (LAI et al., 2013). Also, Type 1-pocket is created by loops of amino acid residues that are mainly neutral or hydrophobic (ERNST et al., 2018).

2.5.2 Heterofunctional supports

A stricter control of enzyme immobilization processes can be achieved using heterofunctional supports, which have several distinct functionalities on its surface and that are able to interact with groups of enzymes under different circumstances (BARBOSA et al., 2013). The properties of the matrix itself can grant the multifunctionality of the support, being ionic or hydrophobic, and in others cases, this may happen by support activation if the spacer arm introduced is not physically inert (BARBOSA et al., 2013; BETANCOR et al., 2006). These spacer arms can connect many groups in the enzyme structure and form an intense multipoint covalent attachment. Some bifunctional reagents containing carboxy or amino groups can be used for this purpose (RAFIEE; REZAEE, 2021), producing a global rigidification of the enzyme structure, which can prevent enzyme inactivation due to conformational changes (BARBOSA et al., 2013). However, some protein areas are more relevant than others, which may render these effects unpredictable (HERNANDEZ; FERNANDEZ-LAFUENTE, 2011).

To achieve intense multipoint covalency, it is necessary to employ supports with large internal surfaces and many reactive groups that can interact with those present under the surface of each protein molecule (BARBOSA et al., 2013). Other important requirements are

low steric hindrance between the enzyme and the supports groups, high stability of supports groups involved with the enzyme reactivity with the support, spacer arms long enough to avoid steric hindrances during the reaction, while still short enough to transmit the rigidity of the support to the enzyme, and an endpoint support that is chemically and physically inert (BARBOSA et al., 2013).

Heterofunctional supports present main groups (or 'primary groups') and also present different secondary groups, which should provide the first immobilization of the enzyme and directs its orientation on this support (MATEO, CESAR et al., 2007). Thus being, heterofunctional supports first adsorb the enzyme by the action of their secondary groups, following by subsequent multipoint covalent attachment with their primary group (MATEO, CESAR et al., 2007). Glutaraldehyde-activated supports are the oldest multifunctional supports (BARBOSA et al., 2013). These supports react mainly with the non-ionized primary amino groups of proteins and are generally used at neutral pHs to immobilize proteins, due to their low stability at alkaline pHs. Immobilization onto these supports may take different pathways. When a high ionic strength is used as the controlling factor, the protein areas showing a high concentration of hydrophobic groups are involved in the first immobilization by hydrophobic adsorption, then the reactive groups in this area may react with the support to form multipoint covalent interactions (BARBOSA et al., 2013). Another possibility is working under low ionic strengths, where firstly, enzyme immobilization by ionic exchange occurs, followed by enzyme nucleophile reactions with glutaraldehyde moieties (BARBOSA et al., 2013).

2.5.2.1 Chitosan

Chitosan is a product of the deacetylation of chitin and presents a mixture of N-acetylglucosamine and glucosamine in its structure (WAHBA, 2020). Some advantages involve biocompatibility, biodegradability, and anti-microbial activity, which allows for different applications (WAHBA, 2020). As a support for enzyme immobilization, it is an example of an ionic matrix that is produced from natural renewable sources in addition to being biodegradable and water-insoluble (SASHIWA; SHIGEMASA, 1999; SHEN et al., 2016). Chitosan is classed as a hydrophilic support due to its primary amino and hydroxyl groups, which are susceptible to chemical modifications and allow for its use in enzyme immobilization protocols via different methods, such as adsorption and covalent bonding (KRAJEWSKA, 2004). However, a chemical modification of chitosan is required if the

enzyme is to be covalently immobilized onto it (MENDES et al., 2013a; RODRIGUES, D. S. et al., 2008; SILVA et al., 2012a). The heterofunctionality process of chitosan comprises two steps: first, its amino groups adsorb the relevant protein, and secondly, a covalent attachment between the protein and the introduced functional group in chitosan is obtained (URRUTIA et al., 2018). The adsorption step is favored by low ionic strengths and pHs, which must be lower than the pKa of the carrier amino groups, but higher than the isoelectric point of the enzyme (URRUTIA et al., 2018).

2.6 Crosslinkers for enzyme immobilization

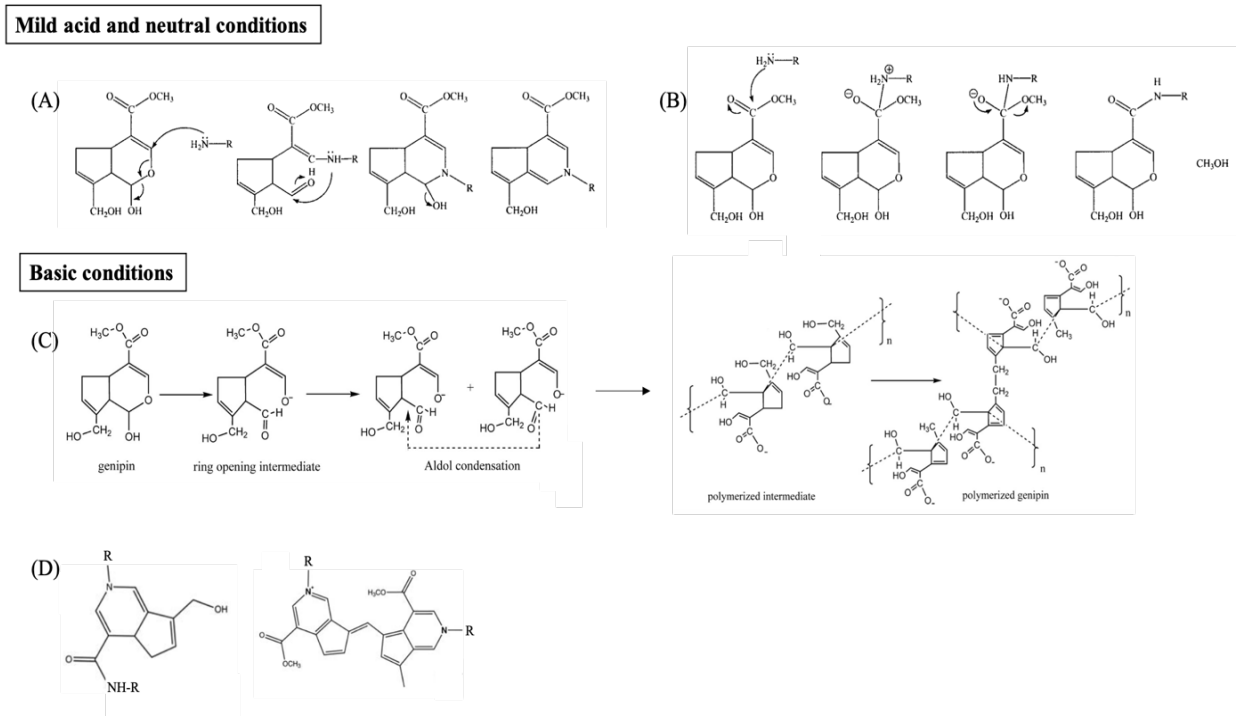
One of the important requisites for obtaining a successful enzyme immobilization protocol that involves covalent attachments is to choose the most ideal reactive groups for the specific application at hand. These groups should show good stability under the proposed reaction conditions, low steric hindrance, and preferably, good reactivity with the amino acids frequently located in the enzyme surface (COWAN; FERNANDEZ-LAFUENTE, 2011). Glutaraldehyde, for instance, is a widely-used crosslinker in the design of biocatalysts, owing to its reactivity with different enzyme moieties (BARBOSA et al., 2014). However, due to its toxicity, others crosslinkers have been studied as alternatives for biocatalyst design (DIMIDA et al., 2015). Genipin, for example, appears as an important replacement for glutaraldehyde due to its lower toxicity, biocompatibility, and ability to cross-link amino acids and proteins (BELLÉ et al., 2018; CUI et al., 2017; DIMIDA et al., 2015). Thus being, a general vision of the potential of genipin and glutaraldehyde, when used as reactive groups for enzyme immobilization, is presented in the following sections.

2.6.1 Genipin

Genipin is obtained from geniposide and presents antioxidant, anti-inflammatory, and antifungal activities, among others (FLORES et al., 2019). It can also enable cross-linking reaction between amino acids and proteins (BELLÉ et al., 2018; CUI et al., 2017) and has shown reduced toxicity compared to other agents, such as glutaraldehyde and formaldehyde (DIMIDA et al., 2015). Supports activated by genipin may be considered heterofunctional supports (TACIAS-PASCACIO et al., 2019), and they enhance the biocompatibility of materials when compared with the materials cross-linked by glutaraldehyde or any other epoxy compound (MANICKAM; SREEDHARAN; ELUMALAI,

2014). Additionally, different forms of cross-linking can be achieved with genipin. pH plays an important role on these reactions, as shown in Figure 6 (DELMAR; BIANCO-PELED, 2015). Under mild acidic and neutral conditions, two reactions can take place: one is the nucleophilic attack of the amino groups of the support on the third carbon (C-3) of genipin, to form an intermediate aldehyde group that opens the dihydropyran ring and results in the aldol condensation of a secondary amino group to form aldehyde groups (Figure 6A) (BUTLER; NG; PUDNEY, 2003; FLORES et al., 2019; MI; SHYU; PENG, 2005; MUZZARELLI, 2009); the other reaction is the nucleophilic attack of amino groups to carboxyl groups of genipin to form a secondary amide (Figure 6B) (DIMIDA et al., 2015). Also, both reactions can occur under these conditions, leading to an absence of exposed groups available for enzymatic binding (Figure 6D) (DIMIDA et al., 2015). At alkaline pHs, the nucleophilic attack of hydroxyl ions (OH^-) occurs due to the genipin molecules opening their rings to form intermediate aldehyde groups (Figure 6C) (MI; SHYU; PENG, 2005). These open-rings monomers then suffer aldol condensation and polymerize to form macromers (MI; SHYU; PENG, 2005). It can be noted that, under alkaline conditions, genipin prioritizes the ring-opening polymerization in detriment of cross-linking with support, leading to long genipin bridges being formed between the polymer chains (MI; SHYU; PENG, 2005).

Figure 6 - (A) Nucleophilic attack of the amino groups of the support on the carbon 3 (C-3) of genipin. (B) Nucleophilic attack of amino groups of the support to carboxyl groups of genipin (C) Nucleophilic attack of hydroxyl ions (OH^-) to genipin molecules. (D) Polymerization of genipin



Source: elaborated by the author adapted from Dimida et al., (2015) and Mi; Shyu; Peng, (2005).

Lower concentrations of genipin lead to less efficient ring activations, and consequently to lower immobilization yields (MA et al., 2018). Higher concentrations of genipin allow for more rings to be activated; however, a parallel reaction between genipin molecules can occur, leading to fewer reaction groups available for enzyme immobilization (MA et al., 2018). Also, cross-linking with genipin at acidic and neutral conditions can lead to the formation of short bridges, while under alkaline conditions, these bridges are longer (DELMAR; BIANCO-PELED, 2015; MI; SHYU; PENG, 2005). Thus being, variations in genipin concentration, pH, time, temperature, and oxygen are parameters that can interfere in the way genipin reacts and polymerizes, and can generate molecules with different spatial structures, colors, and reaction capacities (DIMIDA et al., 2015).

One example of support that is mainly activated with genipin is chitosan, since genipin cross-linking properties decreases its degradation rate (MUZZARELLI, 2009). The activation of chitosan with genipin follows the same scheme suggested above; however, under acidic conditions, the presence of primary amino groups protonated on chitosan leads to a preference for ring-opening reactions by nucleophilic substitution on the ester groups of

genipin to form secondary amides with chitosan (MI; SHYU; PENG, 2005). Under alkaline conditions, genipin molecules form a polymeric network by Schiff bases between genipin and chitosan amino groups (DELMAR; BIANCO-PELED, 2015); however, in the presence of strong bases, chitosan activation with genipin consist of primary chains of chitosan and long cross-link bridges of polymerized genipin (MI; SHYU; PENG, 2005). During these cross-linking reactions, a blue color in the support is expected due to the oxygen radical-induced polymerization of the intermediate genipin compounds (DELMAR; BIANCO-PELED, 2015).

Genipin is a bi-functional compound and, as such, reacts with the support and the proteins involved (especially those containing amino groups) (MUZZARELLI et al., 2016). Supports activated with genipin can immobilize enzymes by covalent, ion exchange, or hydrophobic interactions, which can be controlled by genipin concentration (TACIAS-PASCACIO et al., 2019). A genipin reaction with proteins involves the cross-linking of free amino groups, together with lysine, hydroxylysine, and arginine (MANICKAM; SREEDHARAN; ELUMALAI, 2014). In these circumstances, there is a nucleophilic attack of the primary amine of the protein towards genipin following aldol condensation (WANG; JIANG; XIONG, 2019). Also, the sulfhydryl groups of proteins may be involved in the cross-linking reaction by forming covalent intermolecular disulfide bonds (WANG; JIANG; XIONG, 2019). Under alkaline pHs, the hydroxyl groups of proteins attack the genipin ring, leading to covalent genipin-protein bonds (FLORES et al., 2019). Intramolecular and intermolecular cross-links can be generated by genipin, leading to changes in protein structure such as a reduction of surface hydrophobicity or occlusions of hydrophobic cavities clefs as a result of protein-protein or protein-support association (WANG; JIANG; XIONG, 2019).

2.6.2 Glutaraldehyde

Glutaraldehyde is a bi-functional reagent widely used in the design of biocatalysts owing to the fact it can react with different enzyme moieties, such as primary amino groups, thiols, phenols, and imidazole; however, it also reacts mainly with the primary amino groups of proteins (BARBOSA et al., 2014). The glutaraldehyde structure is not linear, and its main reactive species are in equilibrium regarding their monomeric and polymeric conformations (BARBOSA et al., 2014; KILDEEVA et al., 2009). Each conformation can react in a different manner with proteins and polymers containing amino groups (KILDEEVA et al., 2009). Under acidic and neutral pHs, a nucleophilic attack of the amino groups to glutaraldehyde occur to form Schiff bases (BARBOSA et al., 2013, 2014). Under alkaline pHs, a polymeric

form is produced after an intramolecular aldolic condensation of glutaraldehyde, and the internal aldehyde groups from its polymeric form may react with primary amino groups from proteins or polymers to form Schiff bases (BARBOSA et al., 2013, 2014). However, due to the inconstancy regarding the ratio of equilibrium forms of glutaraldehyde, the exact mechanism of reaction of glutaraldehyde with proteins and polymers are still unknown (KILDEEVA et al., 2009).

Support activation with glutaraldehyde occurs by the modification of primary amino groups of the support by glutaraldehyde (BETANCOR et al., 2006). After activation, the formation of spacer arms bearing one or two amino groups, a hydrophobic moiety formed by glutaraldehyde chain, or covalent reactive groups may occur, but the exact structure formed after the activation is hard to predict (BARBOSA et al., 2013; MIGNEAULT et al., 2004). In this way, the enzyme can interact by hydrophobic, ion exchange, or covalent attachment interactions with the support (BARBOSA et al., 2012). When the support is highly activated, enzyme immobilization can take place by any of these modalities. However, when the support is activated with weakly-activated groups, the enzyme immobilization at neutral pHs will occur first by the terminal amino groups of proteins (if it is exposed), followed by a possible reaction between the amino groups of lysine groups and their support groups, to form multipoint covalent attachments (HERNANDEZ; FERNANDEZ-LAFUENTE, 2011). If the terminal amino groups are not exposed, the reaction may occur by lysine residues. Thus, the multifunctionality of supports activated with glutaraldehyde allows the enzyme to be oriented in different forms by changing immobilization conditions, as well as by provoking a rapid ionic exchange with the support, which prevents the inactivation of soluble enzymes by precipitation (BARBOSA et al., 2013).

Glutaraldehyde-activated chitosan is one of the most widely used supports for enzyme immobilization, and it involves a process that highly depends on pH, since uncharged amine groups are required for the reaction to occur (ADRIANO et al., 2008). Additionally, glutaraldehyde exists in variable forms under variable pHs (WAHBA, 2020). Under acidic conditions, glutaraldehyde can be found in its monomeric linear, cyclic hemiacetal, or oligomeric cyclic forms (WAHBA, 2020). Schiff's bases may be formed by the monomeric linear form of glutaraldehyde and the amino groups of chitosan, while cyclic glutaraldehyde forms will form cyclic derivatives via reactions of the hydroxyl groups with amino groups of chitosan (WAHBA, 2020). In turn, under alkaline pHs, glutaraldehyde exists in its alfa, beta-unsaturated and oligomeric aldehyde forms, which reacts with amino groups of chitosan to form Schiff's bases (WAHBA, 2020). From this perspective, the activation of chitosan with

glutaraldehyde occurs by the formation of covalent bonds by reactions involving the nucleophilic amino groups of chitosan with aldehyde groups of glutaraldehyde (GONÇALVES et al., 2005; WAHBA, 2020). These aldehyde moieties give very reactive aldehyde groups for covalent immobilization, which are very useful even if the high reactivity observed makes the multi-interaction process difficult to control (ADRIANO et al., 2008; WAHBA, 2020).

2.7 Laccase applications

Laccases have been employed in a variety of applications, such as in the modification of food color, elimination of phenols that cause browning and turbidity in beers and wines (RODRÍGUEZ COUTO; TOCA HERRERA, 2006), lignin degradation in the paper industry (CAMARERO et al., 2004), dye decomposition in textile industries (BLÁNQUEZ et al., 2004) and in the fabrication of biosensors (RODRÍGUEZ COUTO; TOCA HERRERA, 2006). Moreover, these enzymes can be applied in the nanobiotechnology, cosmetic, and synthetic chemical industries, apart from also showing potential for the removal of organic micropollutants from water (BA et al., 2018). These enzymes can also catalyze the oxidation of aromatic or phenolic compounds present in wastewater deriving from personal care products (PCPs) and endocrine-disrupting chemicals (EDCs) to form compounds of lower toxicity (BA et al., 2013; JAHANGIRI et al., 2018; PEZZELLA; GUARINO; PISCITELLI, 2015; VISHNU et al., 2017).

In recent years, urbanization and industrialization have brought harmful effects to ecosystems, with serious contaminations being detected in waterbodies (ARREGUI et al., 2019). Studies report that many micropollutants are not entirely eliminated during conventional water treatment and are not biodegraded by the environment either (BILA; DEZOTTI, 2007; MARYŠKOVÁ et al., 2020). Since the concentrations of the pollutants have increased significantly, water quality is currently one of the most relevant societal topics, and the development of innovative tools to tackle this issue is one of the main goals of environmental research (BECKER et al., 2016; CHEN, C. et al., 2018; UPADHYAY; SHRIVASTAVA; AGRAWAL, 2016). In this context, more research focusing on the elimination of micropollutants presented in wastewaters is needed, but the enzymatic route has already emerged as a potential alternative to degrade these micropollutants (BA et al., 2013), which has been shown by current research (CABANA; JONES; AGATHOS, 2009; MARYŠKOVÁ et al., 2020; TOUAHAR et al., 2014; WU et al., 2019).

2.7.1 Removal of organic micropollutants: pharmaceutically active compounds (PhACs)

Pharmaceutical products such as analgesics, anti-inflammatory drugs, antibiotics, and etc., have been used to improve life quality in health treatments, and their consumption is set to increase significantly in future years (COUTO; LANGE; AMARAL, 2019). Pharmaceutically active compounds (PhACs) contaminate water systems due to human excretion (urine and feces), wrongful disposal, landfill leachate, drain water, or from industries (COUTO; LANGE; AMARAL, 2019; NAGHDI et al., 2018b). Some of these compounds, such as acetaminophen and mefenamic acid, have very high usage and greatly affect humans and aquatic organisms (MORSI et al., 2020; NAGHDI et al., 2018b).

Acetaminophen (paracetamol) is an analgesic and antipyretic drug with notable uses owing to the restriction of aspirin as an antipyretic for pregnant women and children. The drug is available since the 1950s and has been one of the most used drugs worldwide (RAMACHANDRAN; JAESCHKE, 2019). Acetaminophen is widely used to treat headaches, fever, aches, and pains (PARKER; COLLETT; WERLER, 2020); however, a link between acetaminophen and asthma was discovered when the medication was taken during pregnancy and childhood (HENDERSON; SHAHEEN, 2013). Additionally, this active chemical has been widely detected in wastewaters in high concentrations, and the effects in humans could escape detection limits, if these are subtle (BA et al., 2014). Aquatic organisms also can be affected even if the drug is present in concentrations in the order of ng/L to µg/L (BA et al., 2014). Therefore, the discharge of acetaminophen in waterbodies, without complete elimination by sewage treatment plants (STP), and its possible interaction with aquatic and human organisms, have stimulated research developments aiming to achieve a complete degradation of this compound (ZHANG, Y.; ZHANG; HONG, 2017). A benzene ring core, substituted by one hydroxyl group, along with the nitrogen atom of an acetamino group, in the para pattern, forms the acetaminophen molecule, which can be oxidized by laccase to form a free radical by the loss of an electron from hydroxyl or amide groups (LU; HUANG; MAO, 2009).

Laccase mediates the one-electron oxidation of acetaminophen, which results in the formation of free acetaminophen radicals as primary products (JAHANGIRI et al., 2018). These radicals couple to each other to form oligomers by covalent bonding of two parent molecules with one hydrogen atom eliminated from each molecule. Also, the bond between these molecules must be triggered by the unsubstituted carbon atoms on the aromatic ring, since the formation of bonds with oxygen and nitrogen atoms are less favorable than with

carbons, due to their higher number of charges (LU; HUANG; MAO, 2009). Furthermore, the increase in the degrees of polymerization tends to form oligomers that are less hydrophilic, allowing for better compound separation from aqueous systems through precipitation, coagulation, or adsorption process.

Mefenamic acid (2-[(2,3-dimethyl phenyl) amino] benzoic acid) is another PhAC that is reasonably difficult to be degraded and that is frequently found in water (MURSHID; DHAKSHINAMOORTHY, 2019). It is a non-steroidal anti-inflammatory drug (NSAIDs) commonly used for pain management, such as pre-menstrual and menstrual cramps, post-operative pain, dysmenorrhea, osteoarthritis, headache, and post-partum pain (MOLL et al., 2011; MURSHID; DHAKSHINAMOORTHY, 2019). Mefenamic acid molecules present amine, carboxylic, and benzylic rings, apart from methyl groups that present low aqueous solubility and high permeability through biological membranes (MUDALIP et al., 2016).

2.7.2 Removal of organic micropollutants: endocrine disrupting chemicals (EDC)

The U.S. Environmental Protection Agency (EPA) defines endocrine-disrupting chemicals (EDCs) as "exogenous agents that interfere with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process." (DIAMANTI-KANDARAKIS et al., 2009). Their mechanism involves nuclear receptors, non-nuclear steroid hormone receptors, non-steroid receptors, neurotransmitter receptors, enzymatic pathways, among others (DE COSTER; VAN LAREBEKE, 2012; DIAMANTI-KANDARAKIS et al., 2009). Some of them, such as xenobiotics and environmental contaminants, can inhibit the activity of histone deacetylase and stimulate the activity of the mitogen-activated protein kinase (TABB; BLUMBERG, 2006). They also influence the activity of peptide and steroid hormones and the increase of obesity, since they affect the modulation of lipid metabolism and adipogenesis (TABB; BLUMBERG, 2006). Moreover, recent reports showed an increment in testicular cancer, undescended testis, declining of semen quality, testicular dysgenesis syndrome, neuroendocrinology, thyroid metabolism, cardiovascular endocrinology, among other issues, all related to the use of EDCs (DE COSTER; VAN LAREBEKE, 2012; DIAMANTI-KANDARAKIS et al., 2009; RODPRASERT et al., 2019)

Compounds such as bisphenol A, triclosan, and nonylphenol are frequently detected in the waterbodies of areas that are intensively urbanized (CABANA; JONES;

AGATHOS, 2007). These chemicals are endocrine-disrupting entities and can interfere in the human endocrine system, affecting important physiological processes involving overall health, growth, and reproduction. They can also cause decreased hatching of bird and fish eggs, alterations in the immune system of marine mammals, reduced sperm count in humans, and increased risks of breast, testicular, and prostate cancer (BILA; DEZOTTI, 2007). These compounds usually have a low molecular mass (<1000 Daltons) and are present in several industries, such as the plastic, pesticide, and fuel (CABANA; JONES; AGATHOS, 2007). However, even though these compounds are not entirely eliminated during conventional water treatments (BILA; DEZOTTI, 2007; MARYŠKOVÁ et al., 2020), studies have demonstrated success in the oxidation of these compounds by other methods, such as by laccase biocatalysts (CABANA; JONES; AGATHOS, 2009; MARYŠKOVÁ et al., 2020; TOUAHAR et al., 2014; WU et al., 2019).

2.8 References

ADDORISIO, V.; SANNINO, F.; MATEO, C.; GUISÁN, J. M. Oxidation of phenyl compounds using strongly stable immobilized-stabilized laccase from Trametes versicolor. **Process Biochemistry**, [*s. l.*], v. 48, n. 8, p. 1174–1180, 2013.

ADRIANO, W.; MENDONÇA, D. B.; RODRIGUES, D. S.; MAMMARELLA, E. J.; GIORDANO, R. L C. Improving the properties of chitosan as support for the covalent multipoint immobilization of chymotrypsin. **Biomacromolecules**, [*s. l.*], v. 9, n. 8, p. 2170–2179, 2008.

ANAH IACUTE, A. T.; ELIZET, C. C.; FRANCISCO, J. F.; ARTURO, E. T.; ALBA, O. M. G. Temporal profiles of intra- and extracellular laccase isoenzymes from Pleurotus ostreatus produced in submerged and solid-state cultures. **African Journal of Biotechnology**, [*s. l.*], v. 15, n. 22, p. 1069–1077, 2016.

ARANA-PEÑA, S.; RIOS, N. S.; MENDEZ-SANCHEZ, C.; LOKHA, Y.; GONÇALVES, L. R. B.; FERNÁNDEZ-LAFUENTE, R. Use of polyethylenimine to produce immobilized lipase multilayers biocatalysts with very high volumetric activity using octyl-agarose beads: Avoiding enzyme release during multilayer production. **Enzyme and Microbial Technology**, [*s. l.*], v. 137, p. 109535, 2020, 2019.

ARREGUI, L.; AYALA, M.; GÓMEZ-GIL, X.; GUTIÉRREZ-SOTO, G.; HERNÁNDEZ-LUNA, C. E.; HERRERA DE LOS SANTOS, M.; LEVIN, L.; ROJO-DOMÍNGUEZ, A.; ROMERO-MARTÍNEZ, D.; SAPARRAT, M. C. N.; TRUJILLO-ROLDÁN, M. A.; VALDEZ-CRUZ, N. A. Laccases: structure, function, and potential application in water bioremediation. **Microbial Cell Factories**, [*s. l.*], v. 18, n. 1, p. 200, 2019.

BA, S.; HAROUNE, L.; SOUMANO, L.; BELLENGER, J.; JONES, J. P.; CABANA, H. A hybrid bioreactor based on insolubilized tyrosinase and laccase catalysis and microfiltration

membrane remove pharmaceuticals from wastewater. **Chemosphere**, [*s. l.*], v. 201, p. 749–755, 2018.

BA, S.; ARSENAULT, A.; HASSANI, T.; JONES, J. P.; CABANA, H. Laccase immobilization and insolubilization: from fundamentals to applications for the elimination of emerging contaminants in wastewater treatment. **Critical Reviews in Biotechnology**, [*s. l.*], v. 33, n. 4, p. 404–418, 2013.

BA, S.; HAROUNE, L.; CRUZ-MORATÓ, C.; JACQUET, C.; TOUAHAR, I. E.; BELLENGER, J.; LEGAULT, C. Y.; JONES, J. P.; CABANA, H. Synthesis and characterization of combined cross-linked laccase and tyrosinase aggregates transforming acetaminophen as a model phenolic compound in wastewaters. **Science of The Total Environment**, [*s. l.*], v. 487, n. 1, p. 748–755, 2014.

BALDRIAN, P. Fungal laccases – occurrence and properties. **FEMS Microbiology Reviews**, [*s. l.*], v. 30, n. 2, p. 215–242, 2006.

BARBOSA, O.; ORTIZ, C.; BERENGUER-MURCIA, Á.; TORRES, R.; RODRIGUES, R. C.; FERNANDEZ-LAFUENTE, R. Glutaraldehyde in bio-catalysts design: a useful crosslinker and a versatile tool in enzyme immobilization. **RSC Adv.**, [*s. l.*], v. 4, n. 4, p. 1583–1600, 2014.

BARBOSA, O.; TORRES, R.; ORTIZ, C.; BERENGUER-MURCIA, A.; RODRIGUES, R. C.; FERNÁNDEZ-LAFUENTE, R. **Heterofunctional supports in enzyme immobilization: From traditional immobilization protocols to opportunities in tuning enzyme properties**. [*s. l.*], 2013.

BARBOSA, O.; ORTIZ, C.; BERENGUER-MURCIA, Á.; TORRES, R.; RODRIGUES, R. C.; FERNANDEZ-LAFUENTE, R. Strategies for the one-step immobilization–purification of enzymes as industrial biocatalysts. **Biotechnology Advances**, [*s. l.*], v. 33, n. 5, p. 435–456, 2015.

BARBOSA, O.; TORRES, R.; ORTIZ, C.; FERNANDEZ-LAFUENTE, R. Versatility of glutaraldehyde to immobilize lipases: Effect of the immobilization protocol on the properties of lipase B from *Candida antarctica*. **Process Biochemistry**, [*s. l.*], v. 47, n. 8, p. 1220–1227, 2012.

BASSANINI, I.; FERRANDI, E. E.; RIVA, S.; MONTI, D. Biocatalysis with laccases: An updated overview. **Catalysts**, [*s. l.*], v. 11, n. 1, p. 26, 2020.

BAYNE, L.; ULIJN, R. V.; HALLING, P. J. Effect of pore size on the performance of immobilised enzymes. **Chem. Soc. Rev**, [*s. l.*], v. 42, n. 22, p. 9000-9010, 2013.

BECKER, D.; VARELA DELLA GIUSTINA, S.; RODRIGUEZ-MOZAZ, S.; SCHOEVAART, R.; BARCELÓ, D.; CAZES, M.; BELLEVILLE, M.; SANCHEZ-MARCANO, J.; GUNZBURG, J.; COUILLEROT, O.; VOLKER, J.; OEHLMANN, J.; WAGNER, M. Removal of antibiotics in wastewater by enzymatic treatment with fungal laccase – Degradation of compounds does not always eliminate toxicity. **Bioresource Technology**, [*s. l.*], v. 219, p. 500–509, 2016.

BELLÉ, A. S.; FERRANDI, E. E.; RIVA, S.; MONTI, D. Efficient enzyme-assisted extraction of genipin from genipap (*Genipa americana L.*) and its application as a crosslinker for chitosan gels. **Food Chemistry**, [*s. l.*], v. 246, p. 266–274, 2018.

BERKA, R. M.; SCHNEIDER, P.; GOLIGHTLY, E. J; BROWN, S. H.; MADDEN, M.; BROWN, K. M.; HALKIER, T.; MONDORF, K.; XU, F. Characterization of the gene encoding an extracellular laccase of myceliophthora thermophila and analysis of the recombinant enzyme expressed in aspergillus oryzae. **Applied and Environmental Microbiology**, [*s. l.*], v. 63, n. 8, p. 3151–3157, 1997.

BETANCOR, L.; LÓPEZ-GALLEGO, F.; HIDALGO, A.; ALONSO-MORALES, N.; MATEO, G. D.; FERNÁNDEZ-LAFUENTE, R.; GUISÁN, J. M. Different mechanisms of protein immobilization on glutaraldehyde activated supports: Effect of support activation and immobilization conditions. **Enzyme and Microbial Technology**, [*s. l.*], v. 39, n. 4, p. 877–882, 2006.

BILA, D. M.; DEZOTTI, M. **Desreguladores endócrinos no meio ambiente: Efeitos e conseqüências**. [*s. l.*], 2007.

BILAL, M.; ASGHER, M.; PARRA-SALDIVAR, R.; HU, H.; WANG, W.; ZHANG, X.; IQBAL, H. M. N. **Immobilized ligninolytic enzymes: An innovative and environmental responsive technology to tackle dye-based industrial pollutants – A review**. [*s. l.*], 2017.

BLÁNQUEZ, P.; CASAS, N.; FONT, X.; GABARRELL, X.; SARRÀ, M.; CAMINAL, G.; VICENT, T. Mechanism of textile metal dye biotransformation by Trametes versicolor. **Water Research**, [*s. l.*], v. 38, n. 8, p. 2166–2172, 2004.

BUTLER, M. F.; NG, Y.; PUDNEY, P. D. A. Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin. **Journal of Polymer Science Part A: Polymer Chemistry**, [*s. l.*], v. 41, n. 24, p. 3941–3953, 2003.

CABANA, H.; JONES, J. P.; AGATHOS, S. N. Preparation and characterization of cross-linked laccase aggregates and their application to the elimination of endocrine disrupting chemicals. **Journal of Biotechnology**, [*s. l.*], v. 132, n. 1, p. 23–31, 2007.

CABANA, H.; JONES, J. P.; AGATHOS, S. N. Utilization of cross-linked laccase aggregates in a perfusion basket reactor for the continuous elimination of endocrine-disrupting chemicals. **Biotechnology and Bioengineering**, [*s. l.*], v. 102, n. 6, p. 1582–1592, 2009.

CAMARERO, S.; GARCÍA, O.; VIDAL, T.; COLOM, J.; DEL RÍO, J. C.; GUTIÉRREZ, A.; GRAS, J. M.; MONJE, R.; MARTÍNEZ, M. J.; MARTÍNEZ, Á. T. Efficient bleaching of non-wood high-quality paper pulp using laccase-mediator system. **Enzyme and Microbial Technology**, [*s. l.*], v. 35, n. 2–3, p. 113–120, 2004.

CAO, L. Immobilised enzymes: science or art? **Current Opinion in Chemical Biology**, [*s. l.*], v. 9, n. 2, p. 217–226, 2005.

CAO, L.; LANGEN, L.; SHELDON, R. A. Immobilised enzymes: carrier-bound or carrier-free? **Current Opinion in Biotechnology**, [*s. l.*], v. 14, n. 4, p. 387–394, 2003.

CARRAWAY, K. L.; KOSHLAND, D. E. Reaction of tyrosine residues in proteins with carbodiimide reagents. **Biochimica et Biophysica Acta (BBA) - Protein Structure**, [*s. l.*], v. 160, n. 2, p. 272–274, 1968.

CARRAWAY, K. L.; TRIPLETT, R. B. Reaction of carbodiimides with protein sulfhydryl groups. **Biochimica et Biophysica Acta (BBA) - Protein Structure**, [*s. l.*], v. 200, n. 3, p. 564–566, 1970.

CHAMPAGNE, P.-P.; NESHEIM, M. E.; RAMSAY, J. A. A mechanism for NaCl inhibition of Reactive Blue 19 decolorization and ABTS oxidation by laccase. **Applied Microbiology and Biotechnology**, [*s. l.*], v. 97, n. 14, p. 6263–6269, 2013.

CHEN, C.; SUN, W.; LV, H.; LI, H.; WANG, Y.; WANG, P. Spacer arm-facilitated tethering of laccase on magnetic polydopamine nanoparticles for efficient biocatalytic water treatment. **Chemical Engineering Journal**, [*s. l.*], v. 350, n. 130, p. 949–959, 2018.

COUTO, C. F.; LANGE, L. C.; AMARAL, M. C. S. Occurrence, fate and removal of pharmaceutically active compounds (PhACs) in water and wastewater treatment plants - A review. **Journal of Water Process Engineering**, [*s. l.*], v. 32, p. 100927, 2019.

COWAN, D. A.; FERNANDEZ-LAFUENTE, R. Enhancing the functional properties of thermophilic enzymes by chemical modification and immobilization. **Enzyme and Microbial Technology**, [*s. l.*], v. 49, n. 4, p. 326–346, 2011.

CUI, C.; CHEN, H.; CHEN, B.; TAN, T. Genipin Cross-Linked Glucose Oxidase and Catalase Multi-enzyme for Gluconic Acid Synthesis. **Applied Biochemistry and Biotechnology**, [*s. l.*], v. 181, n. 2, p. 526–535, 2017.

DE COSTER, S.; VAN LAREBEKE, N. Endocrine-disrupting chemicals: Associated disorders and mechanisms of action. **Journal of Environmental and Public Health**, [*s. l.*], v. 2012, p. 1–52, 2012.

DELMAR, K.; BIANCO-PELED, H. The dramatic effect of small pH changes on the properties of chitosan hydrogels crosslinked with genipin. **Carbohydrate Polymers**, [*s. l.*], v. 127, p. 28–37, 2015.

DIAMANTI-KANDARAKIS, Evanthia *et al.* Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement. **Endocrine Reviews**, [*s. l.*], v. 30, n. 4, p. 293–342, 2009. Disponível em: https://doi.org/10.1210/er.2009-0002

DIMIDA, S.; DEMITRI, C.; BENEDICTIS, V. M.; SCALERA, F.; GERVASO, F.; SANNINO, A. Genipin-cross-linked chitosan-based hydrogels: Reaction kinetics and structure-related characteristics, [*s. l.*], v. 42256, p. 1–8, 2015.

DOMARADZKA, D.; GUZIK, U.; WOJCIESZYŃSKA, D. Biodegradation and biotransformation of polycyclic non-steroidal anti-inflammatory drugs. **Reviews in Environmental Science and Bio/Technology**, [*s. l.*], v. 14, n. 2, p. 229–239, 2015.

DU, J. R.; HSU, L. H.; XIAO, E. S.; GUO, X.; ZHANG, Y.; FENG, X. Using genipin as a "green" crosslinker to fabricate chitosan membranes for pervaporative dehydration of isopropanol. **Separation and Purification Technology**, [*s. l.*], v. 244, p. 116843, 2020.

DURÁN, N.; ROSA, M. A.; D'ANNIBALE, A.; GIANFREDA, L. Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review. **Enzyme and Microbial Technology**, [*s. l.*], v. 31, n. 7, p. 907–931, 2002.

ERNST, H. A.; JORGENSEN, L. J.; BUKH, C.; PIONTEK, K.; PLATTNER, D. A.; OSTERGAARD, L. H.; LARSEN, S.; BJERRUM, M. J. A comparative structural analysis of the surface properties of asco-laccases. **PLOS ONE**, [*s. l.*], v. 13, n. 11, p. 1-27, 2018.

FERNÁNDEZ-FERNÁNDEZ, M.; SANROMÁN, M. Á.; MOLDES, D. Recent developments and applications of immobilized laccase. **Biotechnology Advances**, [*s. l.*], v. 31, n. 8, p. 1808–1825, 2013.

FERNANDEZ-LOPEZ, L.; PEDRERO, S. G.; LOPEZ-CARROBLES, N.; GORINES, B. C.; VIRGEN-ORTÍZ, J. J.; FERNANDEZ-LAFUENTE, R. Effect of protein load on stability of immobilized enzymes. **Enzyme and Microbial Technology**, [*s. l.*], v. 98, p. 18–25, 2017.

FERNANDEZ-LORENTE, G.; GODOY, C. A.; MENDES, A. A.; LOPEZ-GALLEGO, F.; GRAZU, V.; DE LAS RIVAS, B.; PALOMO, J. M.; HERMOSO, J.; FERNANDEZ-LAFUENTE, R.; GUISAN, J. M. Solid-phase chemical amination of a lipase from *Bacillus thermocatenulatus* to improve its stabilization via covalent immobilization on highly activated glyoxyl-agarose. **Biomacromolecules**, [*s. l.*], v. 9, n. 9, p. 2553–2561, 2008.

FILHO, M.; PESSELA, B. C.; MATEO, C.; CARRASCOSA, A. V.; FERNANDEZ-LAFUENTE, R.; GUISÁN, J. M. Reversible immobilization of a hexameric α-galactosidase from Thermus sp. strain T2 on polymeric ionic exchangers. **Process Biochemistry**, [*s. l.*], v. 43, n. 10, p. 1142–1146, 2008.

FLORES, E. E. E.; CARDOSO, F. D.; SIQUEIRA, L. B.; RICARDI, N. C.; COSTA, T. H.; RODRIGUES, R. C.; KLEIN, M. P.; HERTZ, P. F. Influence of reaction parameters in the polymerization between genipin and chitosan for enzyme immobilization. **Process Biochemistry**, [*s. l.*], v. 84, n. June, p. 73–80, 2019.

FUENTES, M.; PESSELA, B. C. C.; MAQUIESE, J. V.; ORTIZ, C.; SEGURA, R. L.; PALOMO, J. M.; ABIAN, O.; TORRES, R.; MATEO, C.; FERNANDEZ-LAFUENTE, R.; GUISAN, J. M. Reversible and strong immobilization of proteins by ionic exchange on supports coated with sulfate-dextran. **Biotechnology Progress**, [*s. l.*], v. 20, n. 4, p. 1134–1139, 2004.

GAMALLO, M.; FUENTES, M.; PESSELA, B. C. C.; MAQUIESE, J. V.; ORTIZ, C.; SEGURA, R. L.; PALOMO, J. M.; ABIAN, O.; TORRES, R.; MATEO, C.; FERNANDEZ-LAFUENTE, R.; GUISAN, J. M. Sequential reactors for the removal of endocrine disrupting chemicals by laccase immobilized onto fumed silica microparticles. **Biocatalysis and Biotransformation**, [*s. l.*], v. 36, n. 3, p. 254–264, 2018.

GARCIA-GALAN, C.; BERENGUER-MURCIA, Á.; FERNANDEZ-LAFUENTE, R.; RODRIGUES, R. C. Potential of different enzyme immobilization strategies to improve

enzyme performance. **Advanced Synthesis & Catalysis**, [*s. l.*], v. 353, n. 16, p. 2885–2904, 2011.

GIANFREDA, L.; XU, F.; BOLLAG, J. M. Laccases: A useful group of oxidoreductive enzymes. **Bioremediation Journal**, [*s. l.*], v. 3, n. 1, p. 1–25, 1999.

GIARDINA, P.; FARACO, V.; PEZZELLA, C.; PISCITELLI, A.; VANHULLE, S.; SANNIA, G. Laccases: a never-ending story. **Cellular and Molecular Life Sciences**, [*s. l.*], v. 67, n. 3, p. 369–385, 2010.

GONÇALVES, V. L.; LARANJEIRA, M. C. M.; FÁVERE, V. T.; PEDROSA, R. C. Effect of crosslinking agents on chitosan microspheres in controlled release of diclofenac sodium. **Polímeros**, [*s. l.*], v. 15, n. 1, p. 6–12, 2005.

GUAN, Z.; LUO, Q.; WANG, H.; CHEN, Y.; LIAO, X. Bacterial laccases: promising biological green tools for industrial applications. **Cellular and Molecular Life Sciences**, [*s. l.*], v. 75, n. 19, p. 3569–3592, 2018.

HATA, T.; KAWAI, S.; OKAMURA, H.; NISHIDA, T. Removal of diclofenac and mefenamic acid by the white rot fungus *Phanerochaete sordida* YK-624 and identification of their metabolites after fungal transformation. **Biodegradation**, [*s. l.*], v. 21, n. 5, p. 681–689, 2010.

HENDERSON, A. J.; SHAHEEN, S. O. Acetaminophen and asthma. **Paediatric Respiratory Reviews**, [*s. l.*], v. 14, n. 1, p. 9–16, 2013.

HERNANDEZ, K.; FERNANDEZ-LAFUENTE, R. Control of protein immobilization: Coupling immobilization and site-directed mutagenesis to improve biocatalyst or biosensor performance. **Enzyme and Microbial Technology**, [*s. l.*], v. 48, n. 2, p. 107–122, 2011.

HOARE, D. G.; KOSHLAND, D. E. A method for the quantitative modification and estimation of carboxylic acid groups in proteins. **The Journal of biological chemistry**, [*s. l.*], v. 242, n. 10, p. 2447–2453, 1967.

HOARE, D. G.; KOSHLAND, D. E. A Procedure for the Selective Modification of Carboxyl Groups in Proteins. **Journal of the American Chemical Society**, [*s. l.*], v. 88, n. 9, p. 2057–2058, 1966.

HOARE, D. G.; OLSON, A.; KOSHLAND, D. E. The reaction of hydroxamic acids with water-soluble carbodiimides. A lossen rearrangement. **Journal of the American Chemical Society**, [*s. l.*], v. 90, n. 6, p. 1638–1643, 1968.

ITOH, T.; TAKAGI, Y. Laccase-catalyzed reactions in ionic liquids for green sustainable chemistry. **ACS Sustainable Chemistry & Engineering**, [*s. l.*], v. 9, n. 4, p. 1443–1458, 2021.

JAHANGIRI, E.; THOMAS, I.; SCHULZE, A.; SEIWERT, B.; CABANA, H.; SCHLOSSER, D. Characterisation of electron beam irradiation-immobilised laccase for application in wastewater treatment. **Science of the Total Environment**, [*s. l.*], v. 624, p. 309–322, 2018.

JANUSZ, G.; PAWLIK, A.; ŚWIDERSKA-BUREK, U.; POLAK, J.; SULEJ, J.; JAROSZ-WILKOŁAZKA, A.; PASZCZYŃSKI, A. Laccase properties, physiological functions, and evolution. **International Journal of Molecular Sciences**, [*s. l.*], v. 21, n. 3, p. 966, 2020.

JESIONOWSKI, T.; ZDARTA, J.; KRAJEWSKA, B. **Enzyme immobilization by adsorption: A review**., [*s. l.*], 2014.

JÚNIOR, W. G. M. **Caracterização, imobilização e aplicação das lipases de *Candida rugosa* e *Geotrichum candidum* produzidas em meio contendo melaço de soja**, 1–124 f. 2017. Universidade Federal de Uberlândia, Uberlândia, 2017.

KALYANI, D.; DHIMAN, S. S.; KIM, H.; JEYA, M.; KIM, W.; LEE, J. K. Characterization of a novel laccase from the isolated *Coltricia perennis* and its application to detoxification of biomass. **Process Biochemistry**, [*s. l.*], v. 47, n. 4, p. 671–678, 2012.

KILDEEVA, N. R.; PERMINOV, P. A.; VLADIMIROV, L. V.; NOVIKOV, V. V.; MIKHAILOV, S. N. About mechanism of chitosan cross-linking with glutaraldehyde. **Russian Journal of Bioorganic Chemistry**, [*s. l.*], v. 35, n. 3, p. 360–369, 2009.

KRAJEWSKA, B. Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. **Enzyme and Microbial Technology**, [*s. l.*], v. 35, n. 2–3, p. 126–139, 2004.

KUMAR, V. V.; CABANA, H. Towards high potential magnetic biocatalysts for on-demand elimination of pharmaceuticals. **Bioresource Technology**, [*s. l.*], v. 200, p. 81–89, 2016.

LAI, C.; PERMINOV, P. A.; VLADIMIROV, L. V.; NOVIKOV, V. V.; MIKHAILOV, S. N. Effect of ABTS on the adsorption of Trametes versicolor laccase on alkali lignin. **International Biodeterioration & Biodegradation**, [*s. l.*], v. 82, p. 180–186, 2013.

LIU, Y. HUANG, L.; GUO, W.; JIA, L.; FU, Y.; GUI, S.; LU, F. Cloning, expression, and characterization of a thermostable and pH-stable laccase from *Klebsiella pneumoniae* and its application to dye decolorization. **Process Biochemistry**, [*s. l.*], v. 53, p. 125–134, 2017.

LÓPEZ-GALLEGO, F. Improved stabilization of chemically aminated enzymes via multipoint covalent attachment on glyoxyl supports. **Journal of Biotechnology**, [*s. l.*], v. 116, n. 1, p. 1–10, 2005.

LU, J.; HUANG, Q.; MAO, L. Removal of acetaminophen using enzyme-mediated oxidative coupling processes: i. reaction rates and pathways. **Environmental Science & Technology**, [*s. l.*], v. 43, n. 18, p. 7062–7067, 2009.

MA, H.; MENG, G.; CUI, B.; SI, J.; DAI, Y. Chitosan crosslinked with genipin as supporting matrix for biodegradation of synthetic dyes: Laccase immobilization and characterization. **Chemical Engineering Research and Design**, [*s. l.*], v. 132, n. 35, p. 664–676, 2018.

MALVIYA, S.; ARVIND; JHA, A.; HETTIARACHCHY, N. Antioxidant and antibacterial potential of pomegranate peel extracts. **Journal of Food Science and Technology**, [*s. l.*], v. 51, n. 12, p. 4132–4137, 2014.

MANICKAM, B.; SREEDHARAN, R.; ELUMALAI, M. 'Genipin' – The natural water soluble cross-linking agent and its importance in the modified drug delivery systems: An overview. **Current Drug Delivery**, [*s. l.*], v. 11, n. 1, p. 139–145, 2014.

MARTÍNEZ, A.; ARVIND; JHA, A.; HETTIARACHCHY, N. **Oxidoreductases on their way to industrial biotransformations**. [*s. l.*], 2017.

MARYŠKOVÁ, M.; SCHAABOVÁ, M.; TOMÁNKOVÁ, H.; NOVOTNÝ, V.; RYSOVÁ, M. Wastewater treatment by novel polyamide/polyethylenimine nanofibers with immobilized laccase. **Water**, [*s. l.*], v. 12, n. 2, p. 588, 2020.

MATE, D. M.; ALCALDE, M. **Laccase engineering: From rational design to directed evolution**, [*s. l.*], 2015.

MATEO, C.; PALOMO, J. M..; FERNANDEZ-LORENTE, G.; GUISAN, J. M.; FERNANDEZ-LAFUENTE, R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. **Enzyme and Microbial Technology**, [*s. l.*], v. 40, n. 6, p. 1451–1463, 2007.

MATEO, C.; ABIAN, O.; FERNANDEZ-LAFUENTE, R.; GUISAN, J. M. Reversible enzyme immobilization via a very strong and nondistorting ionic adsorption on support-polyethylenimine composites. **Biotechnology and Bioengineering**, [*s. l.*], v. 68, n. 1, p. 98–105, 2000.

MATYASH, L. F.; OGLOBLINA, O. G.; STEPANOV, V. M. Modification of carboxyl groups in pepsin. **European Journal of Biochemistry**, [*s. l.*], v. 35, n. 3, p. 540–545, 1973.

MENDES, A. A.; DE CASTRO, H. F.; ANDRADE, G. S. S.; TARDIOLI, P. W.; GIORDANO, R. L. C. Preparation and application of epoxy-chitosan/alginate support in the immobilization of microbial lipases by covalent attachment. **Reactive and Functional Polymers**, [*s. l.*], v. 73, n. 1, p. 160–167, 2013.

MI, F.; SHYU, S.; PENG, C. Characterization of ring-opening polymerization of genipin and pH-dependent cross-linking reactions between chitosan and genipin. **Journal of Polymer Science Part A: Polymer Chemistry**, [*s. l.*], v. 43, n. 10, p. 1985–2000, 2005.

MIGNEAULT, I.; DARTIGUENAVE, C.; BERTRAND, M. J.; WALDRON, K. C. Glutaraldehyde: Behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. **BioTechniques**, [*s. l.*], v. 37, n. 5, p. 790–802, 2004.

MILETIĆ, N.; NASTASOVIĆ, A.; LOOS, K. Immobilization of biocatalysts for enzymatic polymerizations: Possibilities, advantages, applications. **Bioresource Technology**, [*s. l.*], v. 115, p. 126–135, 2012.

MOHAMAD, N. R.; MIGNEAULT, I.; DARTIGUENAVE, C.; BERTRAND, M. J.; WALDRON, K. C. An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. **Biotechnology & Biotechnological Equipment**, [*s. l.*], v. 29, n. 2, p. 205–220, 2015.

MOLL, R.; DERRY, S.; MOORE, R. A.; MCQUAY, H. J. Single dose oral mefenamic acid for acute postoperative pain in adults. **Cochrane Database of Systematic Reviews**, [*s. l.*], n. 3, 2011.

MORENO, A. D.; IBARRA, D.; EUGENIO, M. E.; TOMÁS-PEJÓ, E. Laccases as versatile enzymes: from industrial uses to novel applications. **Journal of Chemical Technology & Biotechnology**, [*s. l.*], v. 95, n. 3, p. 481–494, 2020.

MORSI, R.; BILAL, M.; IQBAL, H. M. N.; ASHRAF, S. Laccases and peroxidases: The smart, greener and futuristic biocatalytic tools to mitigate recalcitrant emerging pollutants. **Science of The Total Environment**, [*s. l.*], v. 714, p. 136572, 2020.

MUDALIP, S. K.; ABU BAKAR, M. R.; JAMAL, P.; ADAM, F.; ALAM, Z. M. Molecular recognition and solubility of mefenamic acid in water. **Asian Journal of Chemistry**, [*s. l.*], v. 28, n. 4, p. 853–858, 2016.

MUNK, L.; SITARZ, A. K.; KALYANI, D. C.; MIKKELSEN, J. D.; MEYER, A. S. Can laccases catalyze bond cleavage in lignin? **Biotechnology Advances**, [*s. l.*], v. 33, n. 1, p. 13–24, 2015.

MURSHID, S.; DHAKSHINAMOORTHY, G. P. Biodegradation of sodium diclofenac and mefenamic acid: Kinetic studies, identification of metabolites and analysis of enzyme activity. **International Biodeterioration & Biodegradation**, [*s. l.*], v. 144, p. 104756, 2019.

MUZZARELLI, R. A. A. Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. **Carbohydrate Polymers**, [*s. l.*], v. 77, n. 1, p. 1–9, 2009.

MUZZARELLI, R. A. A.; EL MEHTEDI, M.; BOTTEGONI, C.; GIGANTE, A. Physical properties imparted by genipin to chitosan for tissue regeneration with human stem cells: A review. **International Journal of Biological Macromolecules**, [*s. l.*], v. 93, p. 1366–1381, 2016.

NAGHDI, M.; TAHERAN, M.; BRAR, S. K.; KERMANSHAHI-POUR, A.; VERMA, M.; SURAMPALLI, R. Y. **Removal of pharmaceutical compounds in water and wastewater using fungal oxidoreductase enzymes**, [*s. l.*], 2018.

NAKAJIMA, N.; IKADA, Y. Mechanism of amide formation by carbodiimide for bioconjugation in aqueous media. **Bioconjugate Chemistry**, [*s. l.*], v. 6, n. 1, p. 123–130, 1995.

NGUYEN, H. H.; KIM, M. An overview of techniques in enzyme immobilization. **Applied Science and Convergence Technology**, [*s. l.*], v. 26, n. 6, p. 157–163, 2017.

NGUYEN, L. N.; HAI, F. I.; PRICE, W. E.; LEUSCH, F. C. D. L.; RODDICK, F.; NGO, H. H.; GUO, W.; MAGRAM, S. F.; NGHIEM, L. D. The effects of mediator and granular activated carbon addition on degradation of trace organic contaminants by an enzymatic membrane reactor. **Bioresource Technology**, [*s. l.*], v. 167, p. 169–177, 2014.

OVERBEEKE, P. L. A.; GOVARDHAN, C.; KHALAF, N.; JONGEJAN, J. A; HEIJNEN, J. J. Influence of lid conformation on lipase enantioselectivity. **Journal of Molecular Catalysis B: Enzymatic**, [*s. l.*], v. 10, n. 4, p. 385–393, 2000.

PARKER, S. E.; COLLETT, B. R.; WERLER, M. M. Maternal acetaminophen use during pregnancy and childhood behavioural problems: Discrepancies between mother- and teacher-reported outcomes. **Paediatric and Perinatal Epidemiology**, [*s. l.*], v. 34, n. 3, p. 299–308, 2020.

PERFETTI, R. B.; ANDERSON, C. D.; HALL, P. L. The chemical modification of papain with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. **Biochemistry**, [*s. l.*], v. 15, n. 8, p. 1735–1743, 1976.

PESSELA, B. C. C.; MUNILLA, R.; BETANCOR, L.; FUENTES, M.; CARRASCOSA, A. V.; VIAN, A.; FERNANDEZ-LAFUENTE, R.; GUISÁN, J. M. Ion exchange using poorly activated supports, an easy way for purification of large proteins. **Journal of Chromatography A**, [*s. l.*], v. 1034, n. 1–2, p. 155–159, 2004.

PESSELA, B.; FUENTES, M.; MATEO, C.; MUNILLA, R.; CARRASCOSA, A. V.; FERNANDEZ-LAFUENTE, R.; GUISAN, J. M. Purification and very strong reversible immobilization of large proteins on anionic exchangers by controlling the support and the immobilization conditions. **Enzyme and Microbial Technology**, [*s. l.*], v. 39, n. 4, p. 909–915, 2006.

PEZZELLA, C.; GUARINO, L.; PISCITELLI, A. How to enjoy laccases. **Cellular and Molecular Life Sciences**, [*s. l.*], v. 72, n. 5, p. 923–940, 2015.

PINAR, O.; KARAOSMANOĞLU, K.; SAYAR, N. A.; KULA, C.; KAZAN, D.; SAYAR, A. A. Assessment of hazelnut husk as a lignocellulosic feedstock for the production of fermentable sugars and lignocellulolytic enzymes. **3 Biotech**, [*s. l.*], v. 7, n. 6, p. 367, 2017.

PINHEIRO, B. B.; RIOS, N. S.; RODRÍGUEZ AGUADO, E.; FERNANDEZ-LAFUENTE, R.; FREIRE, T. M.; FECHINE, P. B. A.; DOS SANTOS, J. C. S.; GONÇALVES, L. R. B. Chitosan activated with divinyl sulfone: a new heterofunctional support for enzyme immobilization. Application in the immobilization of lipase B from Candida antarctica. **International Journal of Biological Macromolecules**, [*s. l.*], v. 130, p. 798–809, 2019.

PLAGEMANN, R.; VON LANGERMANN, J.; KRAGL, U. Microwave-assisted covalent immobilization of enzymes on inorganic surfaces. **Engineering in Life Sciences**, [*s. l.*], v. 14, n. 5, p. 493–499, 2014.

RAFIEE, F.; REZAEE, M. Different strategies for the lipase immobilization on the chitosan based supports and their applications. **International Journal of Biological Macromolecules**, [*s. l.*], v. 179, p. 170–195, 2021.

RAMACHANDRAN, A.; JAESCHKE, H. Acetaminophen hepatotoxicity. **Seminars in Liver Disease**, [*s. l.*], v. 39, n. 02, p. 221–234, 2019.

RE, R.; PELLEGRINI, N.; PROTEGGENTE, A.; PANNALA, A.; YANG, M.; RICE-EVANS, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. **Free Radical Biology and Medicine**, [*s. l.*], v. 26, n. 9–10, p. 1231–1237, 1999.

REIS, C. L. B.; DE SOUSA, E. Y. A.; DE FRANÇA SERPA, J.; OLIVEIRA, R. C.; DOS SANTOS, J. C. S. **Design of immobilized enzyme biocatalysts: Drawbacks and opportunities**. [*s. l.*], 2019.

RIOS, N.; ARANA-PEÑA, S.; MENDEZ-SANCHEZ, C.; LOKHA, Y.; CORTES-CORBERAN, V.; GONÇALVES, L. R. B.; FERNANDEZ-LAFUENTE, R. Increasing the enzyme loading capacity of porous supports by a layer-by-layer immobilization strategy using PEI as glue. **Catalysts**, [*s. l.*], v. 9, n. 7, p. 576, 2019.

RODPRASERT, W.; MAIN, K. M.; TOPPARI, J.; VIRTANEN, H. E. Associations between male reproductive health and exposure to endocrine-disrupting chemicals. **Current Opinion in Endocrine and Metabolic Research**, [*s. l.*], v. 7, p. 49–61, 2019. Disponível em: https://doi.org/10.1016/j.coemr.2019.05.002

RODRIGUES, D. S. Multipoint covalent immobilization of microbial lipase on chitosan and agarose activated by different methods. **Journal of Molecular Catalysis B: Enzymatic**, [*s. l.*], v. 51, n. 3–4, p. 100–109, 2008.

RODRIGUES, R. C.; BARBOSA, O.; ORTIZ, C.; BERENGUER-MURCIA, Á.; TORRES, R.; FERNANDEZ-LAFUENTE, R. Amination of enzymes to improve biocatalyst performance: coupling genetic modification and physicochemical tools. **RSC Adv.**, [*s. l.*], v. 4, n. 72, p. 38350–38374, 2014.

RODRIGUES, R. C.; ORTIZ, C.; BERENGUER-MURCIA, Á.; TORRES, R.; FERNÁNDEZ-LAFUENTE, R. Modifying enzyme activity and selectivity by immobilization. **Chem. Soc. Rev.**, [*s. l.*], v. 42, n. 15, p. 6290–6307, 2013.

RODRÍGUEZ COUTO, S.; TOCA HERRERA, J. L. **Industrial and biotechnological applications of laccases: A review**. [*s. l.*], 2006.

SANTOS, J. C. S.; BARBOSA, O.; ORTIZ, C.; BERENGUER-MURCIA, A.; RODRIGUES, R. C.; FERNANDEZ-LAFUENTE, R. Importance of the Support Properties for Immobilization or Purification of Enzymes. **ChemCatChem**, [*s. l.*], v. 7, n. 16, p. 2413–2432, 2015.

SASHIWA, H.; SHIGEMASA, Y. Chemical modification of chitin and chitosan 2: preparation and water soluble property of N-acylated or N-alkylated partially deacetylated chitins. **Carbohydrate Polymers**, [*s. l.*], v. 39, n. 2, p. 127–138, 1999.

SHEN, X.; SHAMSHINA, J. L.; BERTON, P.; GURAU, G.; ROGERS, R. D. Hydrogels based on cellulose and chitin: fabrication, properties, and applications. **Green Chem.**, [*s. l.*], v. 18, n. 1, p. 53–75, 2016.

SILVA, J. A.; MACEDO, G. P.; RODRIGUES, D. S.; GIORDANO, R. L. C.; GONÇALVES, L. R. B. Immobilization of *Candida antarctica* lipase B by covalent

attachment on chitosan-based hydrogels using different support activation strategies. **Biochemical Engineering Journal**, [*s. l.*], v. 60, p. 16–24, 2012.

SONG, Y.; JIANG, J.; QIN, W.; LI, J.; ZHOU, Y.; GAO, Y. Enhanced transformation of organic pollutants by mild oxidants in the presence of synthetic or natural redox mediators: A review. **Water Research**, [*s. l.*], v. 189, p. 116667, 2021.

SU, J.; FU, J.; WANG, Q.; SILVA, C.; CAVACO-PAULO, A. Laccase: a green catalyst for the biosynthesis of poly-phenols. **Critical Reviews in Biotechnology**, [*s. l.*], v. 38, n. 2, p. 294–307, 2018.

SUNDARAM, U. M.; ZHANG, H. H.; HEDMAN, B.; HODGSON, K. O.; SOLOMON, E. Ipectroscopic investigation of peroxide binding to the trinuclear copper cluster site in laccase: Correlation with the peroxy-level intermediate and relevance to catalysis. **Journal of the American Chemical Society**, [*s. l.*], v. 119, n. 51, p. 12525–12540, 1997.

TABB, M. M.; BLUMBERG, B. New modes of action for endocrine-disrupting chemicals. **Molecular Endocrinology**, [*s. l.*], v. 20, n. 3, p. 475–482, 2006.

TACIAS-PASCACIO, V. G.; GARCÍA-PARRA, E.; VELA-GUTIÉRREZ, G.; VIRGEN-ORTIZ, J. J.; BERENGUER-MURCIA, Á.; ALCÁNTARA, A. R.; FERNANDEZ-LAFUENTE, R. Genipin as an emergent tool in the design of biocatalysts: mechanism of reaction and applications. **Catalysts**, [*s. l.*], v. 9, n. 12, p. 1035, 2019.

TOUAHAR, I. E.; HAROUNE, L.; BA, S.; BELLENGER, J.; CABANA, H. Characterization of combined cross-linked enzyme aggregates from laccase, versatile peroxidase and glucose oxidase, and their utilization for the elimination of pharmaceuticals. **Science of The Total Environment**, [*s. l.*], v. 481, n. 1, p. 90–99, 2014.

TRAN, N. H.; URASE, T.; KUSAKABE, O. Biodegradation characteristics of pharmaceutical substances by whole fungal culture trametes versicolor and its laccase. **Journal of Water and Environment Technology**, [*s. l.*], v. 8, n. 2, p. 125–140, 2010.

UPADHYAY, P.; SHRIVASTAVA, R.; AGRAWAL, P. K. Bioprospecting and biotechnological applications of fungal laccase. **3 Biotech**, [*s. l.*], v. 6, n. 1, p. 15, 2016.

URRUTIA, P.; BERNAL, C.; WILSON, L.; ILLANES, A. Use of chitosan heterofunctionality for enzyme immobilization: β-galactosidase immobilization for galacto-oligosaccharide synthesis. **International Journal of Biological Macromolecules**, [*s. l.*], v. 116, p. 182–193, 2018.

VIRGEN-ORTÍZ, J. J.; SANTOS, J. C. S.; BERENGUER-MURCIA, Á.; BARBOSA, O.; RODRIGUES, R. C.; FERNANDEZ-LAFUENTE, R. **Polyethylenimine: A very useful ionic polymer in the design of immobilized enzyme biocatalysts**. [*s. l.*], 2017.

VISHNU, D.; NEERAJ, G.; SWAROOPINI, R.; SHOBANA, R.; KUMAR, V. V.; CABANA, H. Synergetic integration of laccase and versatile peroxidase with magnetic silica microspheres towards remediation of biorefinery wastewater. **Environmental Science and Pollution Research**, [*s. l.*], v. 24, n. 22, p. 17993–18009, 2017.

WAHBA, M. I. Enhancement of the mechanical properties of chitosan. **Journal of Biomaterials Science, Polymer Edition**, [*s. l.*], v. 31, n. 3, p. 350–375, 2020.

WANG, Q.; JIANG, J.; XIONG, Y. L. Genipin-aided protein cross-linking to modify structural and rheological properties of emulsion-filled hempseed protein hydrogels. **Journal of Agricultural and Food Chemistry**, [*s. l.*], v. 67, n. 46, p. 12895–12903, 2019.

WU, E.; LI, Y.; HUANG, Q.; YANG, Z.; WEI, A.; HU, Q. Laccase immobilization on amino-functionalized magnetic metal organic framework for phenolic compound removal. **Chemosphere**, [*s. l.*], v. 233, p. 327–335, 2019.

XU, F. Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. **Journal of Biological Chemistry**, [*s. l.*], v. 272, n. 2, p. 924–928, 1997.

YANG, J.; LI, W.; BUN NG, T.; DENG, X.; LIN, J.; YE, X. Laccases: Production, expression regulation, and applications in pharmaceutical biodegradation. **Frontiers in Microbiology**, [*s. l.*], v. 8, 2017.

ZAAK, H.; SIAR, E.; KORNECKI, J. F.; FERNANDEZ-LOPEZ, L.; PEDRERO, S. G.; VIRGEN-ORTÍZ, J. J.; FERNANDEZ-LAFUENTE, R. Effect of immobilization rate and enzyme crowding on enzyme stability under different conditions. The case of lipase from Thermomyces lanuginosus immobilized on octyl agarose beads. **Process Biochemistry**, [*s. l.*], v. 56, p. 117–123, 2017.

ZDARTA, J.; MEYER, A. S.; JESIONOWSKI, T.; PINELO, M. **Developments in support materials for immobilization of oxidoreductases: A comprehensive review**. [*s. l.*], 2018.

ZHANG, J.; MEYER, A. S.; JESIONOWSKI, T.; PINELO, M. Layer-by-layer assembly for immobilizing enzymes in enzymatic biofuel cells. **Sustainable Energy & Fuels**, [*s. l.*], v. 4, n. 1, p. 68–79, 2020.

ZHANG, X.; HUA, M.; LV, L.; PAN, B. Ionic Polymer-Coated Laccase with High Activity and Enhanced Stability: Application in the Decolourisation of Water Containing AO7. **Scientific Reports**, [*s. l.*], v. 5, n. 1, p. 8253, 2015. Disponível em: https://doi.org/10.1038/srep08253

ZHANG, Y.; ZHANG, Q.; HONG, J. Sulfate radical degradation of acetaminophen by novel iron–copper bimetallic oxidation catalyzed by persulfate: Mechanism and degradation pathways. **Applied Surface Science**, [*s. l.*], v. 422, p. 443–451, 2017. Disponível em: https://doi.org/10.1016/j.apsusc.2017.05.224

Chapter 3

Laccase amination as a way to improve immobilization via ionic adsorption

3.1 Abstract

The aim of this work was to improve laccase immobilization by enriching protein surface with amination with EDA and EDAC. A laccase previously purified by anion-exchange chromatography and desorbed with NaCl was selected as the model enzyme. Amination of the surface of the purified laccase was performed with ethylenediamine (EDA) and different concentrations of 1-ethyl-3- (3-dimethylamino propyl) carbodiimide (EDAC). Circular Dichroism (CD) spectra of the modified and non-modified laccase suggested an abundance of β-sheet structures and of partial enzyme unfolding with increasing temperature. The results point to the fact that the introduction of amino groups onto the enzyme surface promotes conformational changes that positively affect its secondary structure. However, an increase in amination led to a negative effect in its secondary structure. Subsequently, laccase was immobilized on cationic (sulfopropyl), and anionic (diethylamino ethyl-agarose - DEAE; monoaminoethyl-N-ethyl agarose-MANAE) supports to enable an evaluation of the charges on the enzyme surface. It can be concluded that in both the commercial and the purified extract samples, laccases showed a predominance of negative charges on their surface. These charges, however, become positive after the amination process. The increase in EDAC concentration during amination promoted an increase in the immobilization yield on the sulfopropyl support and a yield decrease on the positively-charged supports. Finally, an evaluation of the stability of the biocatalysts at different pH values (5.0, 7.0, and 9.0) and temperatures (40, 50, and 60 °C) were performed. The results seem to suggest that the enrichment of enzyme surfaces with amino groups is a good strategy to improve industrial laccases via ionic exchange and to increase the efficiency of their immobilization protocols.

Keywords: Amination. Adsorption. CD spectra. Laccase. Stability

3.2 Introduction

There has been an unprecedented drive in the modern chemical industry for biocatalysts that allow for processes that are more economically viable and that meet green chemistry standards of sustainability (BOUDRANT; WOODLEY; FERNANDEZ-LAFUENTE, 2020; SHELDON; WOODLEY, 2018). Intense research in the field of biocatalyst engineering has been carried out to this end and also aiming to solve several other inherent process limitations, such as suboptimal levels of activity, stability, selectivity, specificity, inhibition sensitivity, and chemical resistance (BOUDRANT; WOODLEY; FERNANDEZ-LAFUENTE, 2020; RODRIGUES, R. C. et al., 2013).

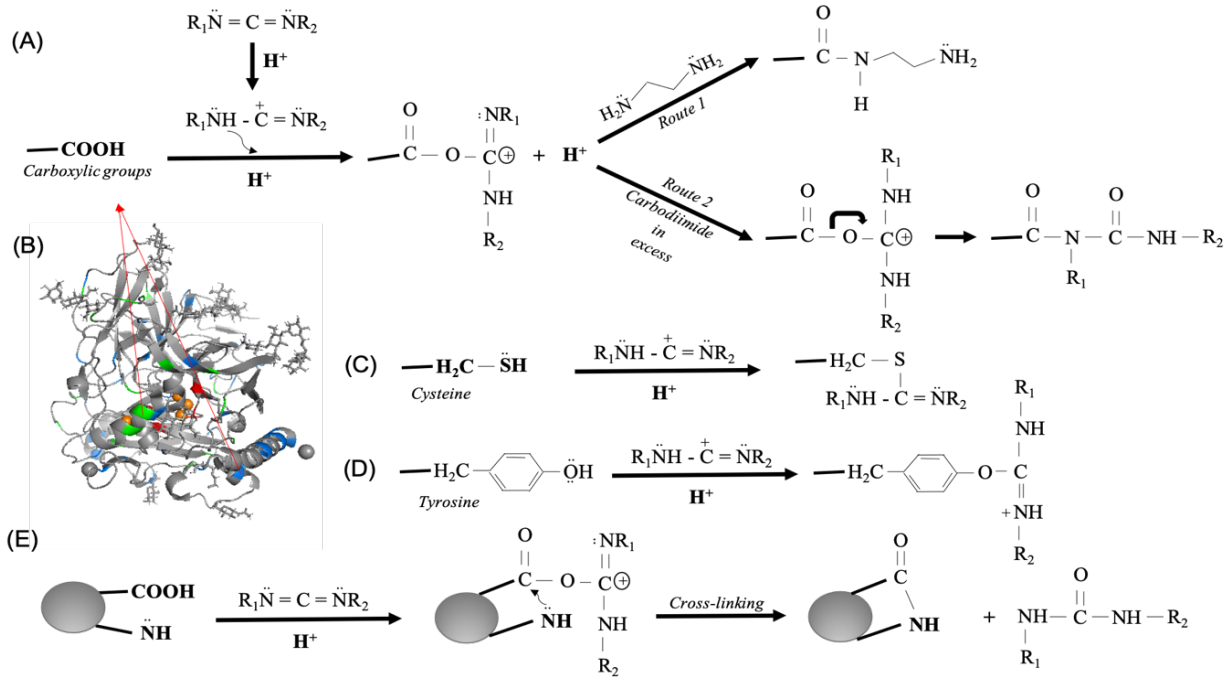
Naturally-obtained enzymes perform their physiological functions under specific conditions of temperature, salinity, and pH (COWAN; FERNANDEZ-LAFUENTE, 2011). Laccase, for example, is an eco-friendly isoenzyme belonging to the group of phenol oxidases that carry out a variety of physiological functions and that can perform well under mild conditions (SU et al., 2018; GIARDINA et al., 2010). These non-substrate-specific enzymes usually possess carbohydrate moieties (mannose, acetylglucosamine, and galactose) that are responsible for their stability (SU et al., 2018). They can catalyze the oxidation of phenolic substrates (RODRÍGUEZ COUTO; TOCA HERRERA, 2006) with only a few requirements, such as the presence of a substrate and oxygen (UPADHYAY; SHRIVASTAVA; AGRAWAL, 2016).

The catalytical mechanisms of laccases involve the four copper atoms that are present in their active site (DURÁN et al., 2002). There, an electron is abstracted from the substrate, the dissolved oxygen is reduced in water, and a free radical is generated (SU et al., 2018). These characteristics render this enzyme suitable and attractive for several applications in different industries (UPADHYAY; SHRIVASTAVA; AGRAWAL, 2016), including the pulp and paper, textile, food, nanobiotechnology, cosmetic, and the synthetic chemistry sectors. The catalyst is also employed in the enzymatic conversion of chemical intermediates, and have shown the potential to remove organic micropollutants from water (BA et al., 2018; JORDAAN et al., 2009; MAYER; STAPLES, 2002; PEZZELLA; GUARINO; PISCITELLI, 2015). In order to streamline the implementation of these potential applications into the industry, laccase immobilization has become a powerful tool for addressing recurring process limitations such as stability loss, high production costs, and the non-reusability of soluble laccases (BRUGNARI et al., 2018).

If well implemented, the immobilization process can increase enzymatic activity and stability, control product formation, and improve the overall process efficiency as well as enzyme recovery (SANTOS et al., 2015). Nevertheless, for this purpose, several aspects must be considered, including the native properties of the support and the active groups present on it, the groups in the enzyme to be immobilized, and the immobilization protocol (BARBOSA et al., 2013). Enzymes from fungi, as is the case with laccases, present a low amount of Lys residues (ADDORISIO et al., 2013), which affects the production of biocatalysts by covalent bonding. Thus being, the chemical modification of these enzymes could be one solution to improve their native properties, apart from being an interesting option for glycoproteins, since the glycosidic chains can mask the amino groups (FERNANDEZ-LAFUENTE et al., 1993).

Chemical amination increases the number of reactive groups on the enzyme surface by introducing amino groups (NH_2^+), as shown in Figure 7. These reactions occur by activating the carboxylic groups of the enzymes with a water-soluble carbodiimide and a modifying reagent (FERNANDEZ-LORENTE et al., 2008; HOARE; KOSHLAND, 1966; HOARE; OLSON; KOSHLAND, 1968; LÓPEZ-GALLEGO et al., 2005; MATYASH; OGLOBLINA; STEPANOV, 1973; PERFETTI; ANDERSON; HALL, 1976). Initially, the 1-ethyl-3-(dimethylamino-propyl) carbodiimide (EDAC) activate the carboxylic groups present, such as the terminal carboxylic, glutamic and aspartic acids (HOARE; KOSHLAND, 1966), by reacting with the hydroxyl groups of these acids to form an intermediate (NAKAJIMA; IKADA, 1995). These reactions occur at pH 4.75, allowing for the dissociation of the carboxylic groups, since ionized carboxyl groups are required in the reaction with EDAC (NAKAJIMA; IKADA, 1995). The activated carboxylic groups then react with the amino groups of a nucleophile, such as ethylenediamine (EDA), to form an amide (HOARE; KOSHLAND, 1967) (Figure 7A, route 1). However, it is important to note that carbodiimides can also react with the free sulfhydryl groups from cysteine (Figure 7C) and the phenolic groups in tyrosine (Figure 7D) at acidic pHs (CARRAWAY; KOSHLAND, 1968; CARRAWAY; TRIPLETT, 1970). The reaction of carbodiimide (EDAC) with sulfhydryl groups presents reaction rates near identical to those of carboxyl groups, while phenolic groups react more slowly (RODRIGUES, R. C. et al., 2014).

Figure 7 - (A) Reactions between the carbodiimide and the carboxyl groups of the protein Novozym 51003. (B) Novozym 51003 structure; copper in the catalytic site is shown as orange spheres, histidine residues are in red, glutamic residues in green, and aspartic residues in blue (PDB code: 6F5K). (C) Reactions between the carbodiimide and sulfhydryl groups of cysteine. (D) Reactions between the carbodiimide and phenolic groups of tyrosine. (E) Intramolecular cross-linking.



Source: elaborated by the author.

The reaction of carbodiimides with the carboxyl group of proteins can follow two pathways, as shown in Figure 7A. Under lower concentrations of carbodiimide, the intermediate product reacts with the amino groups of ethylenediamine to form amide (HOARE; KOSHLAND, 1967) (Figure 7A, route 1). However, in solutions with higher concentrations of carbodiimide, water can also act as a nucleophile, promoting the regeneration of the carboxyl group and converting a molecule of carbodiimide into its corresponding urea (Figure 7A, route 2). This results in enzymatic inhibition (HOARE; KOSHLAND, 1967; NAKAJIMA; IKADA, 1995). Also, other inhibitory effects can occur upon the reaction between carbodiimides and carboxyl groups. These are caused by intramolecular cross-linking between neighboring nucleophiles (RODRIGUES, R. C. et al., 2014) (Figure 7E).

Following the amination process, the newly-introduced amine groups show a lower pKa value than that of lysine and, as a consequence, they are more reactive

(ADDORISIO et al., 2013). The control of the concentration of EDAC in the medium also allows the control of the percentage of modified carboxyl groups (LÓPEZ-GALLEGO et al., 2005). Thus, as the concentration of EDAC in the amination reaction increases, an increase in the introduction of amino groups into the enzyme is expected to occur. This continues until all the superficial carboxylic groups (carboxylic terminal, glutamic, and aspartic acids) have been modified. The immobilization onto anionic (DEAE-agarose and MANAE-agarose) and cationic supports (sulfopropyl) via adsorption allows for an assessment of the amination process. In this technique, key interactions occur between the ionizable groups of the positively- or negatively-charged supports with the functional groups of the enzyme (PESSELA et al., 2006). Some advantages of this process include its flexibility, low cost, support recovery following protein inactivation, process simplicity, rapid immobilization, and very little conformational changes in the enzyme (FUENTES et al., 2004; JESIONOWSKI; ZDARTA; KRAJEWSKA, 2014; REIS et al., 2019).

In this work, we have assessed the possibility of increasing amination levels in laccase by altering the concentration of EDAC in the reaction. Enzyme immobilization yields and stability were also investigated, while circular dichroism analyses predicted its secondary structure conformation and the influence of amination on this. The circular dichroism (CD) spectra of proteins are dependent on their conformation, which enables the monitoring of conformational changes caused by temperature, mutations, denaturants, or binding interactions (GREENFIELD, 2006; WOODY, 1995). The spectroscopic method only show the signals where absorption of radiation occurs, and thus, characteristic peaks are assigned to the distinct structural elements of protein (KELLY; JESS; PRICE, 2005). α-helix proteins, for example, present a positive peak at 198 nm and two negative peaks at 208 nm and 220 nm (LIU, J. et al., 2016), while β-sheet proteins present a negative peak around 218 nm and a positive peak in the range between 185 and 200 nm (XU, P. et al., 2020). An advantage of the use of CD in the study of proteins is that the number of spectral regions can provide different structural information regarding the secondary structure composition, the integrity of cofactor binding sites, conformational changes, and protein folding (KELLY; JESS; PRICE, 2005).

Immobilization by adsorption onto charged supports enables the confirmation of the enzymatic modification and the assessment of the EDAC influence on the amination process. The reason is that the introduction of more amino groups in the molecule of laccase should increase the positive charges on its surface, consequently delivering higher yields of immobilization onto the cationic support and decreasing this yield in the anionic supports. To this end, modified and non-modified laccases were immobilized on anionic and cationic

supports to assess the efficiency of enzyme amination and immobilization, as well as its stability under different pHs and temperatures.

3.3 Materials and methods

3.3.1 Materials

2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), ethylenediamine (EDA), 1-ethyl-3-(3- dimethylamino propyl) carbodiimide (EDAC), and diethylamino ethyl-agarose (DEAE) were purchased from Sigma Aldrich (Brazil). Sigma Chem Co. (Madrid, Spain) supplied the laccase produced by a genetically modified strain of *Aspergillus oryzea* (Novozym 51003) and the sulfopropyl. All the other reagents were of analytical grade.

3.3.2 Methods

3.3.2.1 Preparation of MANAE-agarose

The preparation of glyoxil agarose (4BCL) was carried out as described by Guisán (GUISÁN, 1988). 1.0 g of glyoxyl agarose (4BCL) was suspended in 10 mL of 2 M ethylenediamine at pH 10. The solution was gently stirred for 2 h, filtered and reduced with 10 mg/mL of sodium borohydride ($NaBH_4$). The MANAE-agarose support was washed successively with 100 mM acetate buffer pH 4.0, 100 mM borate buffer pH 9.0, and finally, with distilled water (FERNÁNDEZ-LAFUENTE; RODRIGUEZ; GUISÁN, 1998).

3.3.2.2 Purification of the laccase in DEAE-agarose

Laccase purification was performed using a solution of the commercial enzyme, sodium phosphate buffer pH 7 (5 mM) with 1 mM of copper (II) sulfate pentahydrate, and DEAE-agarose for 1h. Following the DEAE-agarose derivative was suspended in the previous buffer, 120 mM of NaCl, and maintained under agitation for 90 min for the desorption process (VIEIRA et al., 2011). Finally, the purified enzyme was dialyzed for 1h against 5 L of sodium phosphate buffer pH 7 under agitation and using 12-16 KDa cut-off membrane.

3.3.2.3 Chemical amination of purified laccase

The amination was performed adding 4 mL of purified laccase solution (1 U/mL) to 20 mL of ethylenediamine 1M at pH 4.75 and different quantities of solid 1-ethyl-3-(3-dimethylamino propyl) carbodiimide (A1 - 0,024 g; A2 - 0,072 g; A3 - 0,168 g). After 90 min of gentle stirring at 4 °C, the solution was dialyzed against 5 L of 5 mM sodium phosphate buffer pH 7 with 1 mM of copper (II) sulfate pentahydrate for 1 h under gentle stirring (ADDORISIO et al., 2013).

3.3.2.4 Circular Dichroism (CD)

CD spectra over the wavelength range of 190-270 nm were obtained for free commercial laccase (CL), purified laccase (PL), aminated laccase with 0.024 g of EDAC (A1), aminated laccase with 0.072 g of EDAC (A2), and aminated laccase with 0.168 g of EDAC (A3) using a spectrophotometer J-815 CD JASCO (Japan). These laccases were analyzed at a concentration of 0.18 mg/mL (CL), 0.25 mg/mL (EP), 0.23 mg/mL (A1), 0.26 mg/mL (A2), and 0.55 mg/mL (A3) in a 1mm path length cell and at a range of 10-70 °C. The spectrum of each laccase was measured at 25 °C before and after the melting. Sodium phosphate buffer 5 mM pH 7 was used as blank. Analysis of CD data was carried out using the online DICHROWEB website (LOBLEY; WHITMORE; WALLACE, 2002; WHITMORE; WALLACE, 2004, 2008). Each spectrum presented is an average of two measurements (analysis program CONTIN, reference set 7).

3.3.2.5 Immobilization and laccase assay

The immobilizations were carried out using a loading of 5 U/g of modified or non-modified laccases, 5 mM of sodium phosphate buffer pH 7 with 1 mM of copper (II) sulfate pentahydrate (BALDRIAN, 2002; MUTHUVELU et al., 2020), and the support (DEAE-agarose, MANAE-agarose, or sulfopropyl) for 1 hour. All the pH's was adjusted to 7 with a 1 M NaCl or HCl solution.

Laccase activity was determined by oxidation of 1 mL of 3 mM ABTS in sodium acetate buffer (10 mM at pH 4.5), 25μL of enzyme solution or biocatalyst suspension, and 1 mL of sodium acetate buffer. The oxidation of ABTS was followed by measuring the increase

of the absorbance at 418 nm in a 1 cm path length spectrophotometric cell (ε_{ABTS} = 36,000 M^{-1} cm^{-1}) (ADDORISIO et al., 2013). One international unit (IU) of laccase activity corresponds to the oxidation of 1 μmol ABTS per minute under these conditions.

3.3.2.6 Immobilization parameters

The immobilization parameters were calculated according to the literature (BRUGNARI et al., 2018). Immobilization yield (IY) was defined as the ratio between the activity of enzymes retained on the support (At_i – At_f) and the initial solution (At_i). The theoretical immobilized activity (At_R) was calculated by the immobilization yield (IY) versus the enzymatic loading (U/g) used in immobilization. Recovery activity expresses the ratio between the biocatalyst activity (At_D) and the theoretical immobilized activity (At_R) (SILVA et al., 2012).

3.3.2.7 Thermal and pH inactivation of the immobilized enzyme preparations

The effect of pH (5, 7, and 9) and temperature (25, 40, 50, and 60 °C) on the activity of immobilized enzymes was determined using the ABTS assay. The pH conditions were performed with sodium acetate buffer pH 5.0 (5mM), sodium phosphate buffer pH 7.0 (5mM), and sodium carbonate buffer pH 9.0 (5mM) with 1 mM of copper (II) sulfate pentahydrate at 25 °C. All the pH's was adjusted, after biocatalysts addition, with a 1 M NaCl or HCl solution. Thermal stability tests of the immobilized enzymes were performed by incubating enzyme samples at 40, 50, and 60 °C at pH 7.0. Periodically, samples were withdrawn, and the remaining observed activity was measured using the ABTS assay described above. The biocatalysts' half-life ($t_{1/2}$) was calculated according to the Henley & Sadana deactivation model (HENLEY; SADANA, 1985).

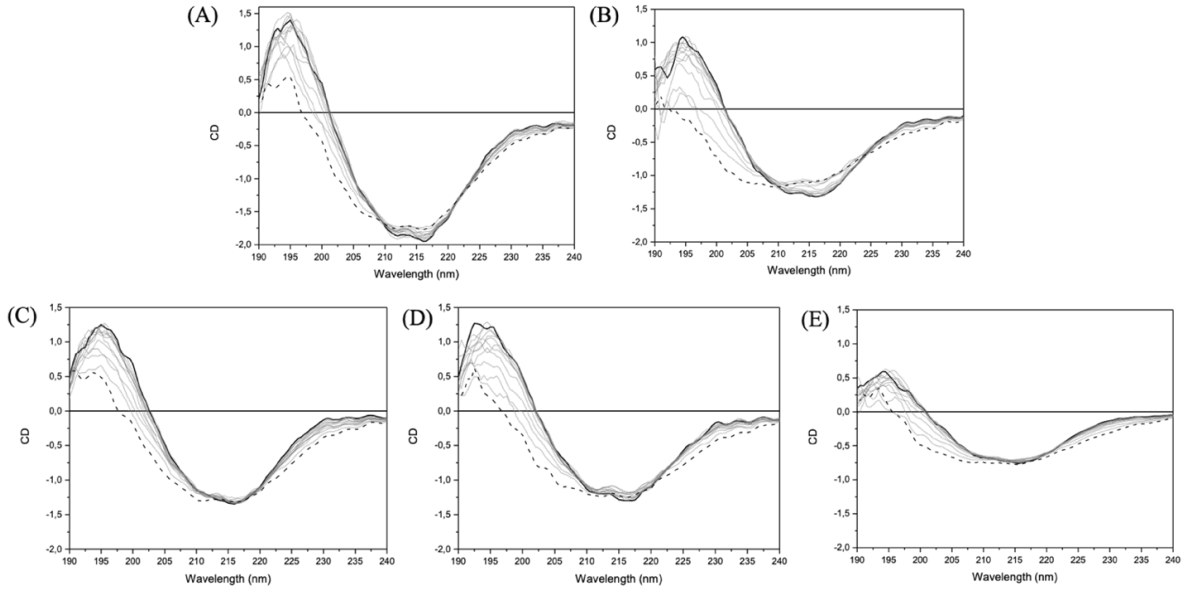
3.4 Results and discussions

3.4.1 Secondary structure of free laccases

CD spectra of proteins can reveal peptide bonds in the far-UV regions when located in a regularly-folded environment, as well as detect changes in an asymmetric

environment or chromophore conformation (LIU, Y. et al., 2017). Thus, the CD spectrum can estimate a protein's secondary structure and determine whether it is correctly folded. Secondary structures are interesting sources of information regarding the mechanisms that dictate important properties, such as temperature and pH optima, thermal stability, and pH stability. In this work, the possible temperature mechanisms of modified and unmodified laccase (Novozym 51003) were analyzed to better understand their structure-function properties under different conditions. CD analyses were performed in the absence of substrates and at temperatures from 10 °C to 70 °C (Figure 8).

Figure 8 - (A) CD spectra of free commercial laccase, (B) free purified laccase, (C) A1: free laccase aminated with 0.024 g of EDAC, (D) A2: free laccase aminated with 0.072 g of EDAC, (E) and A3: free laccase aminated with 0.168 g of EDAC, in the temperature range from 10 °C to 70 °C. The spectrum at 10 °C is represented by a continuous black line, spectra from 15 °C to 65 °C, by a continuous grey line, and the spectrum at 70 °C, by a dotted black line.



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CD spectra of modified and unmodified free laccases (Figure 8) showed that these enzymes contained both α-helix and β-sheet structures belonging to the α + β category (LIU, Y. et al., 2017), which corroborates findings from previous works (BUKH; BJERRUM, 2010; LIU, J. et al., 2016; MUKHOPADHYAY, A.; DASGUPTA; CHAKRABARTI, 2015). The positive peak around 198 nm and the two negative peaks, at around 208 nm and 220 nm, are

both typical of α-helices (LIU, J. et al., 2016). Also, the negative peak at around 218 nm and the positive range between 185 and 200 nm are characteristics of β-fold peaks (XU, P. et al., 2020). Salas et al. (2019) reported a similar spectra for the Novozym 51003, also with the two peaks that are typical of antiparallel β-sheet proteins.

The changes in the CD spectra in Figure 8 suggested that some modifications in the conformation of the modified and unmodified laccases have occurred with the increase of temperature. As it increased, signals became less pronounced for the beta structures, indicating that structures with lower folding degrees had formed (LI et al., 2018). In other words, these results suggest that partial unfolding occurs slowly, but none of the laccases can be said to have completely unfolded. It is also possible to observe that the α-helix and β-sheet structures were more affected by temperature increase in purified laccases than in the commercial laccase extracts (Figure 8A and 8B). This behavior can be explained by the additives present in the commercial laccase, which are used to protect and preserve the enzyme (PISZKIEWICZ; PIELAK, 2019).

A positive effect in the CD spectrum was observed after amination of the purified laccase (PL), which presented negative peaks with a slight signal deviation, and positive peaks with a less intense decay than the purified laccase (Figure 8C). It is well known that the introduction of amino groups into laccase can enhance its rigidity and stability (DE MORAIS JÚNIOR et al., 2017). However, the amination protocol can promote conformational changes in the enzyme (RODRIGUES, R. C. et al., 2014), which can explain the negative effect caused by the increase of amination in its secondary structure (Figure 8D and 8E).

β-sheet arrangements are considered essential antiparallel structures that incorporate copper during catalysis (CHEN, C. et al., 2018). This is a crucial factor to maintain the active central conformation of the enzyme (QIU et al., 2020) and, in general, fungal laccases present several of these arrangements in their secondary structure (MAINARDI et al., 2018). Accordingly, Schneider et al. (1999) reported that the content of secondary structure of a *Coprinus cinereus* laccase was made up by 6% α-helices and 40% β-sheets. Conversely, this content can be different for some fungal laccases, e.g., *Pycnoporus laccases* (3% α-helix and 50% β-sheet) (LIU, J. et al., 2016) and *Coriolus versicolor laccase* (68% α-helix) (QUE et al., 2014). The content of the secondary structure of the laccases investigated in this work is presented in Table 1. The commercial laccase extract (CL) and the purified laccase (PL) at 25 °C showed 8% and 7% of α-helices and 36.5% and 35.1% of β-sheets, respectively, which corroborates the data from previous studies.

Table 1 - Secondary structure content and specific activity (U/mg) of free commercial laccase (CL), purified laccase (PL), laccase aminated with 0.024 g of EDAC (A1), laccase aminated with 0.072 g of EDAC (A2), and laccase aminated with 0.168 g of EDAC (A3), at temperatures of 25 °C, 35 °C, 45 °C, and 70 °C.

Enzyme	**Secondary structure**	**Temperature**		
		25 °C	45 °C	70 °C
CL	Helix (%)	8.0 ± 0.2	8.7 ± 0.8	9.2 ± 0.1
	Strand (%)	36.5 ± 1.1	34.0 ± 0.7	31.0 ± 1.2
PL	Helix (%)	7.0 ± 0.1	7.9 ± 0.0	6.0 ± 0.1
	Strand (%)	35.1 ± 0.4	34.3 ± 0.4	32.8 ± 0.0
A1	Helix (%)	7.0 ± 0.9	7.6 ± 0.4	7.8 ± 0.2
	Strand (%)	36.8 ± 0.2	35.4 ± 0.2	32.7 ± 0.3
A2	Helix (%)	6.7 ± 0.1	7.6 ± 0.4	8.5 ± 0.4
	Strand (%)	36.4 ± 0.6	35.5 ± 0.1	31.4 ± 0.1
A3	Helix (%)	5.7 ± 0.1	6.0 ± 0.0	6.7 ± 0.4
	Strand (%)	35.4 ± 0.4	35.0 ± 0.1	32.5 ± 0.6

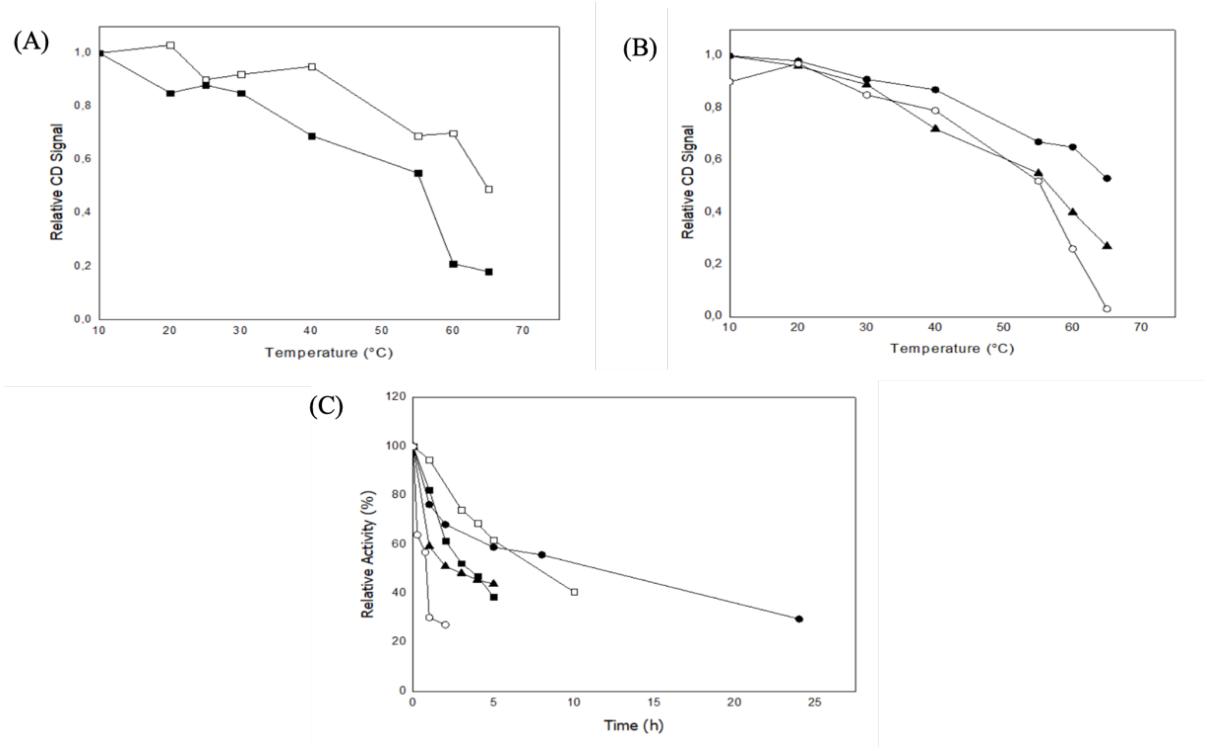
Source: elaborated by the author.

As reported in the literature, when the optimum temperature of an enzyme is exceeded, the hydrogen bond in its helical structure is destroyed as temperature increases (XU, P. et al., 2020). Therefore, the ratio of α-helix structures decreases gradually as they change into β-sheets, β-turns, and random coils, increasing proportion of these structures instead (XU, P. et al., 2020). According to the calculation results (Table 1), the proportion of β-sheets decreased with increasing temperature, while the proportion of α-helices increased, as also reported by Xu et al. (2020) (XU, P. et al., 2020). This result indicates that the optimum temperature was not exceeded in the experiments. The results from Table 1 show that at 25 °C, the β-sheet content of the commercial laccase extract (CL) and the purified laccase (PL) was 36.5% and 35.1%, respectively, with a decrease in these contents as temperature increased, which is indicative of partial unfolding. A decline in β-sheet proportions is also observed with the increase of amination. This has probably occurred due to the fact that ionic interactions on the protein surface are altered with chemical amination (RODRIGUES, R. C. et al., 2014). Consequently, ionic bridges may have broken and been

changed by repulsion forces, possibly affecting the enzyme conformation, as more amino groups were introduced (RODRIGUES, R. C. et al., 2014).

Protein thermostability is related to several physicochemical factors such as protein packing, increased helical fold content, the density of internal hydrogen bonds, distribution of charged residues on the surface, among others (HILDÉN; HAKALA; LUNDELL, 2009; KUMAR, S.; NUSSINOV, 2001; STERNER; LIEBL, 2001). In order to compare the effect of temperature in the modified and unmodified laccases, the significant differences in magnitude of the 195-nm peaks in the CD signals, and the thermal inactivation at 60 °C and pH 7.0, were measured for each laccase, as presented in Figure 9.

Figure 9 - (A) Comparison of thermal denaturations of free commercial laccase (□), free purified laccase (■), data showing the magnitude of the peak at 195nm. (B) Comparison of thermal denaturations of A1: free laccase aminated with 0.024 g of EDAC (●), A2: free laccase aminated with 0.072 g of EDAC (▲), and A3: free laccase aminated with 0.168 g of EDAC (○), data showing the magnitude of the peak at 195 nm . (C) Thermal denaturation of the free commercial laccase (□), free purified laccase (■), A1: free laccase aminated with 0.024 g of EDAC (●), A2: free laccase aminated with 0.072 g of EDAC (▲), and A3: free laccase aminated with 0.168 g of EDAC (○) at 60 °C and pH 7.0.



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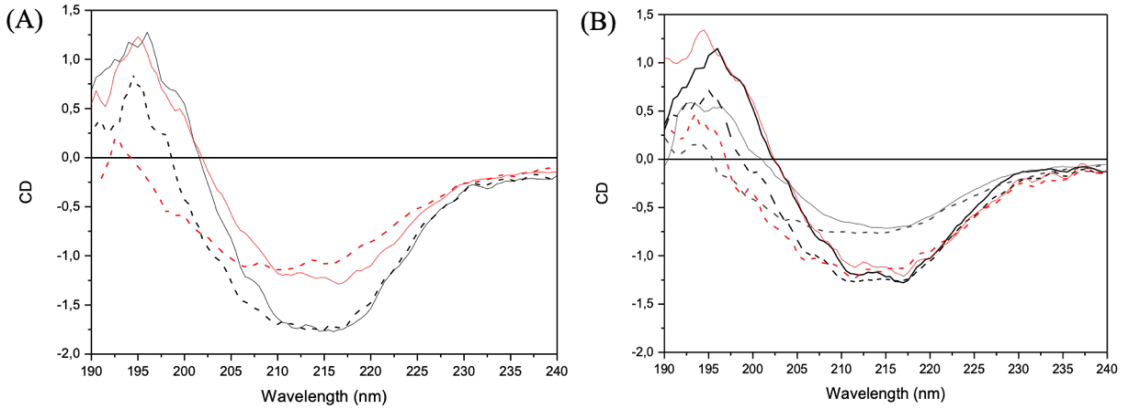
In the case of the free commercial (□) and the free purified laccase (■), the differences in the peak magnitudes of the measured CD signals suggest that the commercial laccase is more thermostable than its purified counterpart at all temperatures tested (Figure 9A). The experimental results corroborate this trend, since the commercial laccase had a half-life time of 7.6 ± 0.52 h, while the purified enzyme, of 3.4 ± 0.2 h at 60 °C and pH 7.0 (Figure 9C). As previously mentioned, this behavior is most likely explained by the protective effects exerted by the additives added to the commercial extracts (PISZKIEWICZ; PIELAK, 2019).

Also, the differences in the peak magnitudes of the measured CD signals of aminated laccases suggest that an increase in EDAC concentration during the amination process led to a negative effect on laccase thermostability. Figure 9B suggests that A1 is more thermostable than A2 and A3 at all temperatures. Experimental results of thermal stability corroborate this trend by confirming that A1 ($t_{1/2}$ = 5.9 ± 0.03 h, at 60 °C and pH 7.0) is more thermostable than A2 ($t_{1/2}$ = 2.6 ± 0.24 h, at 60 °C and pH 7.0) and A3 ($t_{1/2}$ = 0.8 ± 0.0 h, at 60 °C and pH 7.0). This result is expected, since laccase amination with a higher concentration of carbodiimide can lead to more pronounced conformational changes and consequently, to a loss of stability. These results also corroborate previous studies that reported changes in the physical features of the enzyme surface after chemical amination, which can promote conformational changes (RODRIGUES, R. C. et al., 2014). Thus, in order to analyze whether these conformational changes promote a permanent loss in the laccase structure, the CD spectra of modified and unmodified laccases before and after the melting (denaturing) process were performed. These results are reported in Figure 10.

Figure 10 – (A) CD spectra for the free commercial (black) and purified (red) laccase, before (continuous line) and after (dotted line) melting at 70°C. (B) CD spectra for A1: free laccase aminated with 0.024 g of EDAC (black), A2: free laccase aminated with 0.072 g of EDAC (red), and A3: free laccase aminated with 0.168 g of EDAC (grey), before (continuous line) and after (dotted line) melting at 70°C.



Source: elaborated by the author.

The CD spectra before and after melting for the modified and unmodified laccase suggest that the structure of these enzymes are sensitive to elevated temperatures, and these changes in conformation can lead to a loss of enzyme activity (HILDÉN; HAKALA; LUNDELL, 2009). After submitting the free commercial laccase to a temperature of 70 °C, the biocatalyst renatured almost entirely, while the purified enzyme showed a significant damage in its structure under the same conditions, resulting in activity losses (MUKHOPADHYAY, M.; BANERJEE, 2015) (Figure 10A). This behavior can be explained by the higher quantities of additives present in the commercial laccase, such as sorbitol, glucose, glycine, and others (BERKA et al., 1997) that are used to preserve the commercial enzyme (VIEIRA et al., 2011). Similar behavior to that of commercial laccase was observed in the weakly aminated laccase (A1), which was almost entirely renatured after submitting it to the temperature of 70 °C. However, a further increase in the number of amino groups into the laccase structure negatively affected its renaturation after submission to 70 °C. As such, a greater loss in the enzyme structure was observed as more amino groups were introduced (Figure 10B), which leads us to conclude that a lower degree of amination should enhance the secondary structure of laccase. Moreover, the partial thermal inactivation observed in the modified and unmodified laccases was reversible with a high renaturation ratio for the commercial laccase and the less aminated laccase (A1).

3.4.2 Parameters of immobilization of non-modified laccase

At this stage, assays to verify the influence of the additives present in the commercial laccase (CL), as well as the influence of the support charge on the immobilization process, were performed. For this purpose, an immobilization was carried out using 5 U/g of the commercial (CL) or purified laccase (PL) into charged supports. The purification procedure influenced the laccase immobilization parameters as well as the charge of the support. These results are reported in Table 2.

Table 2 - Immobilization Yield (IY), observed activity (At_d) and recovery activity (At_r) of commercial and purified laccase immobilized at different supports

Support	**Purified laccase**			**Commercial laccase**		
	IY (%)	**At_d (U/g)**	**At_r (%)**	**IY (%)**	**At_d (U/g)**	**At_r (%)**
DEAE-agarose	98.8 ± 0.5	4.3 ± 0.1	91.1 ± 1.8	95.9 ± 0.7	3.1 ± 0.1	67.7 ± 2.5
MANAE-agarose	98.4 ± 0.6	2.2 ± 0.0	47.4 ± 0.3	99.7 ± 0.3	2.4 ± 0.1	49.6 ± 1.5
Sulfopropyl	0.0 ± 0.0*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0*	0.0 ± 0.0	0.0 ± 0.0

**Immobilization time in sulfopropyl: 24h.*
Source: elaborated by the author.

From the results in Table 2, a higher enzymatic affinity for anionic supports compared to cationic supports was observed. Once the laccases are immobilized onto anionic supports, a high yield of immobilization is expected since DEAE and MANAE agarose supports are known to adsorb a large number of proteins at pH 7.0 (MATEO, CESAR et al., 2000). Also, enzymes are usually immobilized onto these supports by the region containing the highest concentration of negative charges (VIEIRA et al., 2011). Thus being, the high yield obtained in the anionic supports can be attributed to the predominance of negative charges in the laccase surface due to the aspartic and glutamic acid residues (ROTH; SPIESS, 2015).

Corroborating this hypothesis, the immobilization of laccase on sulfopropyl yielded no immobilization. This agrees with the findings from the literature, where laccases are hardly reported to be adsorbed onto cationic supports due to their isoelectric point being found at pHs 4 to 5 (ERNST et al., 2018; RODRIGUES, R. C. et al., 2014). As such, the lack of affinity for cationic supports can be attributed to the low number of positively-charged groups, such as amino groups, on the surface of laccase (DE MORAIS JÚNIOR et al., 2017).

Additionally, laccases are glycoproteic enzymes in which the glycosidic chains can mask the amino groups (FERNANDEZ-LAFUENTE et al., 1993), preventing them from interacting with the support.

Higher activity recovery was achieved with the DEAE-bound biocatalysts compared to the MANAE-bound biocatalysts. This difference can be due to the high reactivity of laccase residues with MANAE groups, resulting in a distortion in the laccase structure that affects their observed activity (At_d) (BENASSI et al., 2013; PESSELA et al., 2006). Also, DEAE-agarose is a commercial support that possess many ionic groups (PESSELA et al., 2004). This may allow for a better accommodation of the laccase without significant changes to its tridimensional structure and, additionally, to easier access of substrates to the active site of the enzyme.

Commercial laccase extracts also showed high affinity for anionic supports. However, the purified laccase immobilized onto DEAE-agarose presented a higher recovered activity compared with commercial laccase 91.14 ± 1.82 % and 67.71 ± 2.54 %, respectively. The additives used to protect the commercial laccase (glycine, PEG, glucose, among others) (VIEIRA et al., 2011) could explain this behavior, since amino acids and sugars are also included, and these can influence the conformation adopted during immobilization (PISZKIEWICZ; PIELAK, 2019). Nevertheless, the activity of the purified and commercial laccase did not present any differences when immobilized onto MANAE-agarose. This can be due to the high degree of interaction between the groups of MANAE and laccases, which results in a distortion in the enzyme structure (PESSELA et al., 2006), even in the presence of additives. Finally, to analyze the actual immobilization behavior of the laccases, a chemical amination process using the purified laccase extract was studied.

3.4.3 Parameters of immobilization of aminated laccase

The amino groups of proteins show good reactivity with many reagents, which renders the reaction with these groups one of the most applied strategies for immobilization (RODRIGUES, R. C. et al., 2014). It is known that a very wide range of chemicals groups can be introduced into the enzyme structure by chemical modification without requiring previous knowledge of the protein structure (COWAN; FERNANDEZ-LAFUENTE, 2011). Laccases usually have a low amount of Lys residues (ADDORISIO et al., 2013), and the chemical amination processes enriches its surface with low pKa amino groups (around 9.2) compared to that of Lys groups (around 10.7) (FERNANDEZ-LORENTE et al., 2008). The introduction

of amino groups into laccase was performed in this study at three concentrations of EDAC during the amination process. All the aminated laccases (A1, A2, and A3) were immobilized on DEAE-agarose (positive) and sulfopropyl (negative) supports. The results are shown in Table 3.

Table 3 - Immobilization yield (IY) and observed activity (At_d) of aminated laccase (A1, A2, and A3) immobilized onto DEAE-agarose (DA1, DA2, and DA3) and sulfopropyl (SA1, SA2, and SA3) supports.

Biocatalyst	**DA1**	**DA2**	**DA3**	**SA1**	**SA2**	**SA3**
Immobilization Yield (%)	38.2 ± 0.1	46.9 ± 0.2	27.6 ± 4.0	51.8 ± 1.3	62.7 ± 2.3*	64.8 ± 1.4*
Biocatalyst activity (U/g)	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.8 ± 0.05	1.4 ± 0.0*	1.4 ± 0.0*

**Values present no statistically significant differences ($p > 0.05$; $F < F_{critical}$).*

Source: elaborated by the author.

Amino groups are believed to easily interact with sulfopropyl ionic groups (DE MORAIS JÚNIOR et al., 2017). In order to confirm this hypothesis, the aminated laccases (with positive charges) were immobilized on sulfopropyl (negatively charged support). From the results in Table 2, it can be noted that the purified laccase did not become immobilized on sulfopropyl. However, Table 3 shows that the amination process, in fact, allows for the immobilization of laccase on sulfopropyl, apart from an increase in biocatalyst yield and activity from the biocatalyst SA1 to the biocatalyst SA2. This is related to the introduction of more positive charges in the laccase structure, allowing for an increase in their interaction with the sulfopropyl support (negative support) (DE MORAIS JÚNIOR et al., 2018). On the other hand, comparing the parameters of SA2 and SA3, a statistically significant difference ($p > 0.05$) is not observed. This suggests that most of the exposed glutamic and aspartic residues of laccases should be aminated using the second concentration of EDAC (A2). Also, it is clear that while an increase in concentration allows for the introduction of more amino groups, it does not improve the immobilization performance. Finally, as previously noticed in Figure 7, the excess of carbodiimide in the enzyme amination result in the production of a carbodiimide-corresponding urea, which is not an active group and that can trigger intra-

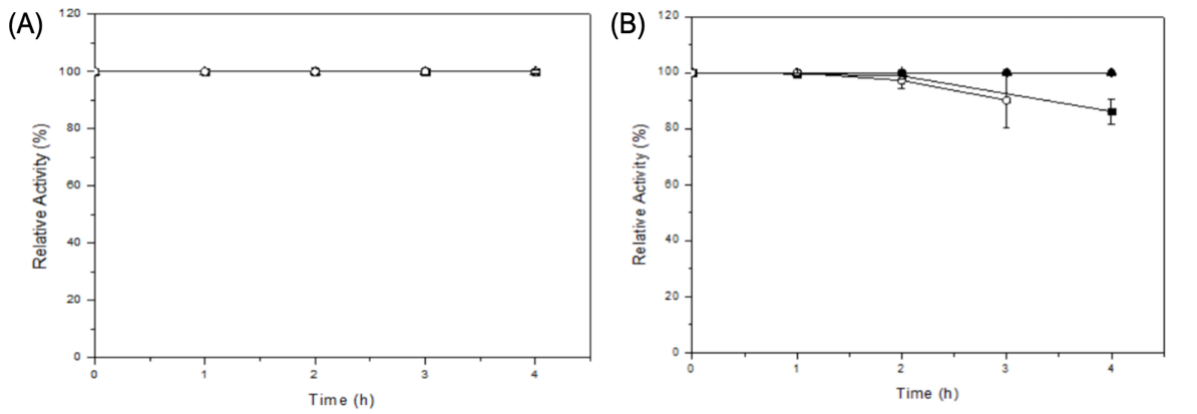
molecular cross-linking interactions that result in enzymatic inhibition (HOARE; KOSHLAND, 1967; NAKAJIMA; IKADA, 1995; RODRIGUES, R. C. et al., 2014).

A drastic decrease in the immobilization yield of DEAE-agarose supports can be observed after laccase amination, which can be observed by comparing Tables 2 and 3. This may be linked to the fact that chemical amination promotes global changes to the physical features of the enzyme surface, such as the alteration of its isoelectric point and of its chemical reactivity (RODRIGUES, R. C. et al., 2014). The yield of the purified laccase immobilized in DEAE-agarose was 98.8 ± 0.5 % (Table 2), indicating a predominance of negative charges on its surface. After amination, there was a significant drop in yield (38.2 ± 0.1 %) for the biocatalyst produced with the lower concentration of EDAC during amination (DA1). This can be explained by the introduction of amino groups on the laccase structure, which in turn, allows the presence of a higher number of cationic groups (positive charges) on their surface and, as a consequence, impairs immobilization onto the DEAE-agarose support (the positively-charged support) (DE MORAIS JÚNIOR et al., 2018). Apart from the decrease in the immobilization yield, some proteins may have still remained attached to the DEAE-agarose support, probably via hydrophobic adsorption (ZUCCA; FERNANDEZ-LAFUENTE; SANJUST, 2016).

3.4.4 Stability of the biocatalysts

To analyze the performance of the biocatalysts, a study on the pH and thermal stabilities of the purified laccase immobilized in DEAE-agarose (PLDEAE) and MANAE-agarose (PLMANAE) was performed. The results are shown in Figure 11 and Table 4.

Figure 11 - (A) Stability of purified laccase immobilized in DEAE-agarose (PLDEAE) at ■ pH 5.0 and 25 °C, • pH 7.0 and 25 °C, ▲ pH 7.0 and 40 °C and ○ pH 7.0 and 50 °C. (B) Stability of purified laccase immobilized in MANAE-agarose (PLMANAE) at ■ pH 5.0 and 25 °C, • pH 7.0 and 25 °C, ▲ pH 7.0 and 40 °C and ○ pH 7.0 and 50 °C.



Source: elaborated by the author.

Figure 11 shows that both biocatalysts are stable at pH 5.0 (25 °C) and pH 7.0 (25, 40, and 50 °C), which could be due to the intramolecular salt bridges between the anionic groups of laccase and the supports, which enhances biocatalyst stability (KILINÇ; ÖNAL; TELEFONCU, 2002). The purified laccase immobilized in DEAE-agarose (PLDEAE) retained 100 % of its activity in all conditions tested (Figure 11A), while the purified laccase immobilized in MANAE-agarose (PLMANAE) presented an activity decay at pH 5.0 (25 °C) and pH 7.0 (50 °C). This must be due to the high reactivity of laccase residues with MANAE-agarose groups (BENASSI et al., 2013; PESSELA et al., 2006), which causes more negative impacts on the biocatalyst when it is exposed to extreme environmental conditions. This could result in distortions to the laccase structure. Moreover, the biocatalysts produced by this technique can desorb from the support when exposed to strong variations in the medium conditions such as pH (PESSELA et al., 2004). pH variations, for example, alter the concentrations of H^+ and OH^- ions in the medium, influencing the ionic interactions between the enzyme and the support.

Table 4 shows the influence of high pH and temperatures in the stability of these biocatalysts. The purified laccase, when immobilized in DEAE, presented higher stability at pH 9.0 than when immobilized in MANAE (Table 4). This can be related to the MANAE support which, once exposed to alkaline pH, presents one positive charge, and in neutral or acid pH, presents a double positive charge (PESSELA et al., 2006). The support behavior at

alkaline pH probably affected the bidimensional interaction between the laccase and the carrier, diminishing the laccase rigidity and, thus, its stability (SANTOS et al., 2015). Also, anions such as CN^-, F^-, CL^- and OH^- can inhibit laccase activity by binding to type II/type III copper and interrupt the internal electron transfer from copper type I to type II/type III center (LIU, Y. et al., 2017).

Table 4 - Stability of purified laccase biocatalysts in sodium carbonate buffer at pH 9.0 at 25 ℃. Thermal stability of purified laccase biocatalysts in sodium phosphate buffer at pH 7.0 at 60 ℃

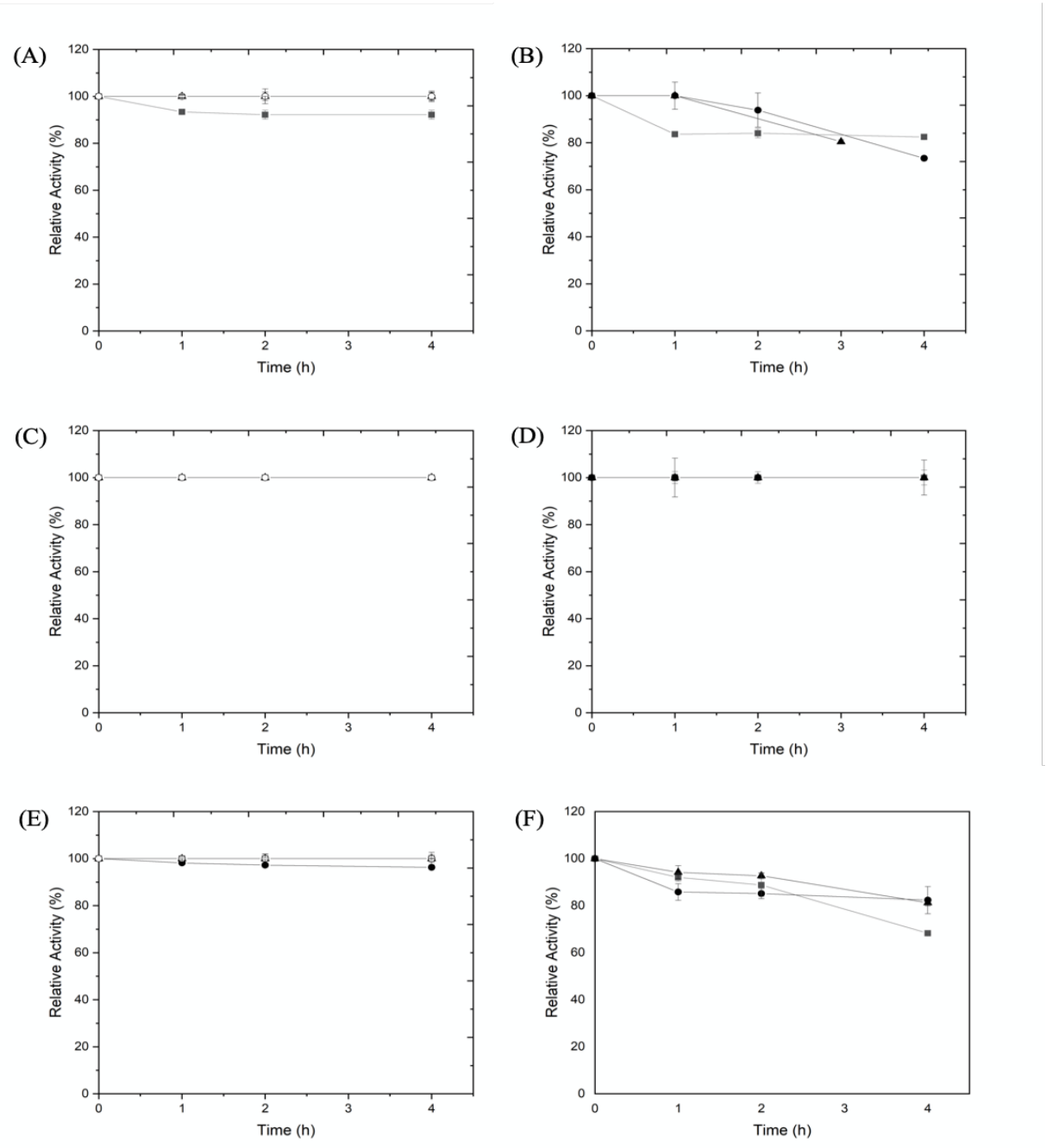
Temperature (℃)	**pH**	**$t_{1/2}$ (h)**	
		PLDEAE	**PLMANAE**
25	9	> 8	1.3 ± 0
60	7	4.1 ± 0.1*	4.6 ± 0.1*

**Values present no statistically significant differences ($p > 0.05$; $F < F_{critical}$).*

Source: elaborated by the author.

No statistically significant differences ($p > 0.05$) were observed between the half-life time (h) of PLDEAE and PLMANAE, at pH 7.0 and 60 ℃. This indicates that both biocatalysts show a similar behavior, except at high pHs (Table 4). Also, both biocatalysts had better half-life times, under the same conditions, than the free purified laccase ($t_{1/2}$ = 3.5 h, measured by fast immobilization in BrCN in one enzyme-support attachment). This may have occurred because laccase was immobilized in both supports by multipoint adsorption (MATEO, CESAR et al., 2000), which allows for more interactions between the groups of the support and of the enzyme, increasing the strength of adsorption and restricting its conformational mobility after immobilization (CRISTÓVÃO et al., 2011). In order to investigate whether the amination process could show an effect on laccase stability, stability assays in the soluble aminated laccases (A1, A2, and A3) and the biocatalysts produced with these aminated laccases on sulfopropyl (SA1, SA2, and SA3) were performed. The results are shown in Figure 12.

Figure 12 - Stability of free purified enzyme (■), A1 (●), A2 (▲), and A3 (○) at (A) pH 5, (C) pH 7, and (E) pH 9 at 25 °C. Stability of SA1 (■), SA2 (●), and SA3 (▲) at (B) pH 5, (D) pH 7 and (F) pH 9 at 25 °C



Source: elaborated by the author.

Laccases from fungi are generally stable under acidic conditions (KASHEFI; BORGHEI; MAHMOODI, 2019). Accordingly, the free purified laccase presented good stability at pH 5.0, which was improved after the amination process (Figure 12A). The

aminated laccases (● A1, ▲ A2, and ○ A3) maintained 100% of their activities after 4 h, while the activity of free purified laccase (■) decreased by around 8% under the same conditions. This can be owing to the amination process, which alters the ionic interactions on the protein surface, affecting its stability, activity, specificity, and selectivity (RODRIGUES, R. C. et al., 2014). When the aminated laccases were immobilized in sulfopropyl, a decay in their stability at pH 5.0 is observed, and the biocatalysts SA1, SA2, and SA3 maintained around 80% of their initial activity (Figure 12B). A similar performance is observed at pH 9.0 with around 80% of the biocatalyst's initial activity remaining after 4h (Figure 12F). This loss in stability is probably due to variations in pH (H^+ and OH^-) that affect the interactions between laccase and sulfopropyl, generating a distortion in laccase conformation and consequently causing its denaturation (CRISTÓVÃO et al., 2011).

Additionally, even if enzyme amination is a powerful tool in the prevention of enzyme desorption (RODRIGUES, R. C. et al., 2014), the presence of unmodified carboxylic groups can promote a reduction in the adsorption strength (MONTES et al., 2006), leading to desorption. Accordingly, a lower activity was detected in the supernatant during laccase denaturation, which seems to indicate laccase desorption (results not shown). The biocatalyst SA3 and the soluble aminated laccase A3 were analyzed for 24h at pH 9.0, which maintained 79.0 ± 0.9 % and 87.0 ± 1.2 % of their initial activity, respectively. Thus, even with the biocatalyst showing signs of denaturation in the first hour (approximately 20%), it could maintain its activity for 24 h. This result indicates that laccase remains active on the support without suffering any further pH effects. Confirming this, Figure 12C and 12D show that at pH 7.0, all the soluble laccases and the biocatalysts maintained 100% of their activity. Finally, to better assess the performance of the biocatalysts, their thermal stability was tested, with the results shown in Table 5.

Table 5 - Thermal stability ($t_{1/2}$, min) of the biocatalysts at 40, 50, and 60 ℃

$t_{1/2}$ (min)		Biocatalyst		
Temperature (℃)	**pH**	**SA1**	**SA2**	**SA3**
40	7	114.1 ± 19.0	> 5 h	> 5 h
50	7	142.0 ± 8.6	331.2 ± 4.2	> 5 h
60	7	59.0 ± 3.8	78.0 ± 1.4	90.0 ± 4.5
60	9	29.0 ± 2.5	52.0 ± 0.5*	59.0 ± 1.6*

**Values present no statistically significant differences ($p > 0.05$; $F < F_{critical}$).*

Source: elaborated by the author.

It was already expected that the sulfopropyl geometry would enable interactions with the amine groups introduced in the enzyme by ion exchange, generating a resistant biocatalyst (DE MORAIS JÚNIOR et al., 2017) and corroborating the results in Table 5. An increase in laccase amination positively affected the biocatalysts' thermal stability, since the biocatalyst SA1 was less stable than SA2 and SA3 at 60 ℃, pH 7.0 and pH 9.0. Furthermore, the biocatalyst SA3 presented an improvement in thermal stability compared to SA2 (60 ℃, pH 7.0). This improvement may be related to a significant increase in the intense multipoint adsorption of laccase to the support by chemical amination, improving the enzyme rigidity (DE MORAIS JÚNIOR et al., 2017). Comparing the biocatalysts' thermal stability at pH 7.0 and 9.0, a decay is observed with pH increase. This result agrees with the literature, since laccase activity can be inhibited by anions such OH^- (LIU, Y. et al., 2017).

The biocatalysts produced with the aminated laccase (Table 5) showed lower stability than those produced with the purified laccase (Table 4). This loss in stability can be explained by laccase desorption and repulsions promoted by unmodified Asp and Glu residues, which have the same charge of the support (sulfopropyl) and can promote a reduction in adsorption strength (MONTES et al., 2006). Also, during the amination process, an alteration in the existing interactions between the groups in the surface of laccases can occur, with ionic bridges being broken and changed by repulsion forces (RODRIGUES, R. C. et al., 2014). This behavior may negatively impact the modified laccase biocatalysts when these are exposed to extreme environments more than they affect unmodified laccases. One study reported that enzyme amination could decrease enzyme stability by up to a 5-fold factor (RODRIGUES, R. C. et al., 2009).

Although the aminated biocatalysts' stability can be lower than the biocatalysts produced with the purified enzyme, the purpose of this work was to analyze the introduction of amino groups in the laccase by changing the EDAC concentration. The results showed significant differences between chemical amination protocols allowing protein surface enriched in reactive groups, giving better options for immobilization protocols with better yielding and increasing the enzyme-support multipoint covalent attachment intensity (LÓPEZ-GALLEGO et al., 2005; RUEDA et al., 2016).

3.5 Conclusions

Increase the concentration of EDAC in the laccase amination process has proved to be a valid strategy to produce protein surfaces enriched with more reactive groups. CD analysis suggests positive effects on laccase secondary structure using the lower amination protocol. An increase in EDAC concentration during amination negatively affects laccase secondary structure, activity, and stability. However, better performance of biocatalysts stability was achieved for the biocatalysts prepared with the higher aminated laccase (SA3). Additionally, surface enrichment with amino groups improves multipoint adsorption of laccase with the support and consequently laccase rigidity. Nevertheless, in the build of biocatalysts, the costs and the additional step of chemical amination should be balanced against the process gains.

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3.7 References

ADDORISIO, V.; SANNINO, F.; MATEO, C.; GUISÁN, J. M. Oxidation of phenyl compounds using strongly stable immobilized-stabilized laccase from Trametes versicolor. **Process Biochemistry**, [*s. l.*], v. 48, n. 8, p. 1174–1180, 2013.

BA, S.; HAROUNE, L.; SOUMANO, L.; BELLENGER, J.; JONES, J. P.; CABANA, H. A hybrid bioreactor based on insolubilized tyrosinase and laccase catalysis and microfiltration membrane remove pharmaceuticals from wastewater. **Chemosphere**, [*s. l.*], v. 201, p. 749–755, 2018.

BALDRIAN, P. Copper and cadmium increase laccase activity in *Pleurotus ostreatus*. **FEMS Microbiology Letters**, [*s. l.*], v. 206, n. 1, p. 69–74, 2002.

BARBOSA, O.; TORRES, R.; ORTIZ, C.; BERENGUER-MURCIA, A.; RODRIGUES, R. C.; FERNÁNDEZ-LAFUENTE, R. **Heterofunctional supports in enzyme immobilization: From traditional immobilization protocols to opportunities in tuning enzyme properties**. [*s. l.*], 2013.

BENASSI, V. M. H. Immobilization and biochemical properties of a β-xylosidase activated by glucose/xylose from Aspergillus niger USP-67 with transxylosylation activity. **Journal of Molecular Catalysis B: Enzymatic**, [*s. l.*], v. 89, p. 93–101, 2013.

BERKA, R. M. Characterization of the gene encoding an extracellular laccase of myceliophthora thermophila and analysis of the recombinant enzyme expressed in aspergillus oryzae. **Applied and Environmental Microbiology**, [*s. l.*], v. 63, n. 8, p. 3151–3157, 1997.

BOUDRANT, J.; WOODLEY, J. M.; FERNANDEZ-LAFUENTE, R. Parameters necessary to define an immobilized enzyme preparation. **Process Biochemistry**, [*s. l.*], v. 90, p. 66–80, 2020.

BRUGNARI, T.; PEREIRA, M. G.; BUBNA, G. A.; FREITAS, E. N.; CONTATO, A. G.; CORRÊA, R. C. G.; CASTOLDI, R.; SOUZA, C. G. M.; POLIZELI, M. L. T. M.; BRACHT, A.; PERALTA, R. M. A highly reusable MANAE-agarose-immobilized Pleurotus ostreatus laccase for degradation of bisphenol A. **Science of the Total Environment**, [*s. l.*], v. 634, p. 1346–1351, 2018.

BUKH, C.; BJERRUM, M. J. The reversible depletion and reconstitution of a copper ion in Coprinus cinereus laccase followed by spectroscopic techniques. **Journal of Inorganic Biochemistry**, [*s. l.*], v. 104, n. 10, p. 1029–1037, 2010.

CARRAWAY, K. L.; KOSHLAND, D. E. Reaction of tyrosine residues in proteins with carbodiimide reagents. **Biochimica et Biophysica Acta (BBA) - Protein Structure**, [*s. l.*], v. 160, n. 2, p. 272–274, 1968.

CARRAWAY, K. L.; TRIPLETT, R. B. Reaction of carbodiimides with protein sulfhydryl groups. **Biochimica et Biophysica Acta (BBA) - Protein Structure**, [*s. l.*], v. 200, n. 3, p. 564–566, 1970.

CHEN, C.; SUN, W.; LV, H.; LI, H.; WANG, Y.; WANG, P. Spacer arm-facilitated tethering of laccase on magnetic polydopamine nanoparticles for efficient biocatalytic water treatment. **Chemical Engineering Journal**, [*s. l.*], v. 350, n. 130, p. 949–959, 2018.

COWAN, D. A.; FERNANDEZ-LAFUENTE, R. Enhancing the functional properties of thermophilic enzymes by chemical modification and immobilization. **Enzyme and Microbial Technology**, [*s. l.*], v. 49, n. 4, p. 326–346, 2011.

CRISTÓVÃO, R. O. Immobilization of commercial laccase onto green coconut fiber by adsorption and its application for reactive textile dyes degradation. **Journal of Molecular Catalysis B: Enzymatic**, [*s. l.*], v. 72, n. 1–2, p. 6–12, 2011.

DE MORAIS JÚNIOR, W. G. Influence of different immobilization techniques to improve the enantioselectivity of lipase from Geotrichum candidum applied on the resolution of mandelic acid. **Molecular Catalysis**, v. 458, n. July, p. 89–96, 2018.

DE MORAIS JÚNIOR, W. G.; TERRASAN, C. R. F.; FERNÁNDEZ-LORENTE, G.; GUISÁN, J. M.; RIBEIRO, E. J.; RESENDE, M. M.; PESSELA, B. C. Solid-phase amination of Geotrichum candidum lipase: ionic immobilization, stabilization and fish oil hydrolysis for the production of Omega-3 polyunsaturated fatty acids. **European Food Research and Technology**, [*s. l.*], v. 243, n. 8, p. 1375–1384, 2017.

DE SALAS, F. Engineering of a fungal laccase to develop a robust, versatile and highly-expressed biocatalyst for sustainable chemistry. **Green Chemistry**, [*s. l.*], v. 21, n. 19, p. 5374–5385, 2019.

DICHROWEB. **On-line analysis for protein Circular Dichroism spectra**, 2021. Available in: <http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>. Accessed on: 11 may 2021

DURÁN, N.; ROSA, M. A.; D'ANNIBALE, A.; GIANFREDA, L. Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review. **Enzyme and Microbial Technology**, [*s. l.*], v. 31, n. 7, p. 907–931, 2002.

ERNST, H. A.; JORGENSEN, L. J.; BUKH, C.; PIONTEK, K.; PLATTNER, D. A.; OSTERGAARD, L. H.; LARSEN, S.; BJERRUM, M. J. A comparative structural analysis of the surface properties of asco-laccases. **PLOS ONE**, [*s. l.*], v. 13, n. 11, p. 1-27, 2018.

FERNANDEZ-LAFUENTE, R. Preparation of activated supports containing low pK amino groups. A new tool for protein immobilization via the carboxyl coupling method. **Enzyme and Microbial Technology**, [*s. l.*], v. 15, n. 7, p. 546–550, 1993.

FERNÁNDEZ-LAFUENTE, R.; RODRIGUEZ, V.; GUISÁN, J. M. The coimmobilization of d-amino acid oxidase and catalase enables the quantitative transformation of d-amino acids (d-phenylalanine) into α-keto acids (phenylpyruvic acid). **Enzyme and Microbial Technology**, [*s. l.*], v. 23, n. 1–2, p. 28–33, 1998.

FERNANDEZ-LORENTE, G.; GODOY, C. A.; MENDES, A. A.; LOPEZ-GALLEGO, F.; GRAZU, V.; DE LAS RIVAS, B.; PALOMO, J. M.; HERMOSO, J.; FERNANDEZ-LAFUENTE, R.; GUISAN, J. M. Solid-phase chemical amination of a lipase from *Bacillus thermocatenulatus* to improve its stabilization via covalent immobilization on highly activated glyoxyl-agarose. **Biomacromolecules**, [*s. l.*], v. 9, n. 9, p. 2553–2561, 2008.

FUENTES, M.; PESSELA, B. C. C.; MAQUIESE, J. V.; ORTIZ, C.; SEGURA, R. L.; PALOMO, J. M.; ABIAN, O.; TORRES, R.; MATEO, C.; FERNANDEZ-LAFUENTE, R.; GUISAN, J. M. Reversible and strong immobilization of proteins by ionic exchange on supports coated with sulfate-dextran. **Biotechnology Progress**, [*s. l.*], v. 20, n. 4, p. 1134–1139, 2004.

GIARDINA, P.; FARACO, V.; PEZZELLA, C.; PISCITELLI, A.; VANHULLE, S.; SANNIA, G. Laccases: a never-ending story. **Cellular and Molecular Life Sciences**, [*s. l.*], v. 67, n. 3, p. 369–385, 2010.

GREENFIELD, N. J. Using circular dichroism spectra to estimate protein secondary structure. **Nature Protocols**, [*s. l.*], v. 1, n. 6, p. 2876–2890, 2006.

GUISÁN, J. M. Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes. **Enzyme and Microbial Technology**, [*s. l.*], v. 10, n. 6, p. 375–382, 1988.

HENLEY, J. P.; SADANA, A. **Categorization of enzyme deactivations using a series-type mechanism**. [*s. l.*], 1985.

HILDÉN, K.; HAKALA, T. K.; LUNDELL, T. Thermotolerant and thermostable laccases. **Biotechnology Letters**, [*s. l.*], v. 31, n. 8, p. 1117–1128, 2009.

HOARE, D. G.; KOSHLAND, D. E. A method for the quantitative modification and estimation of carboxylic acid groups in proteins. **The Journal of biological chemistry**, [*s. l.*], v. 242, n. 10, p. 2447–2453, 1967.

HOARE, D. G.; KOSHLAND, D. E. A procedure for the selective modification of carboxyl groups in proteins. **Journal of the American Chemical Society**, [*s. l.*], v. 88, n. 9, p. 2057–2058, 1966.

HOARE, D. G.; OLSON, A.; KOSHLAND, D. E. The reaction of hydroxamic acids with water-soluble carbodiimides. A lossen rearrangement. **Journal of the American Chemical Society**, [*s. l.*], v. 90, n. 6, p. 1638–1643, 1968.

JESIONOWSKI, T.; ZDARTA, J.; KRAJEWSKA, B. **Enzyme immobilization by adsorption: A review**. [*s. l.*], 2014.

JORDAAN, J.; MATHYE, S.; SIMPSON, C.; BRADY, D. Improved chemical and physical stability of laccase after spherezyme immobilisation. **Enzyme and Microbial Technology**, [*s. l.*], v. 45, n. 6–7, p. 432–435, 2009.

KASHEFI, S.; BORGHEI, S. M.; MAHMOODI, N. M. Covalently immobilized laccase onto graphene oxide nanosheets: Preparation, characterization, and biodegradation of azo dyes in colored wastewater. **Journal of Molecular Liquids**, [*s. l.*], v. 276, p. 153–162, 2019.

KELLY, S. M.; JESS, T. J.; PRICE, N. C. How to study proteins by circular dichroism. **Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics**, [*s. l.*], v. 1751, n. 2, p. 119–139, 2005.

KILINÇ, A.; ÖNAL, S.; TELEFONCU, A. Stabilization of papain by modification with chitosan. **Turkish Journal of Chemistry**, [*s. l.*], v. 26, n. 3, p. 311–316, 2002.

KUMAR, S.; NUSSINOV, R. How do thermophilic proteins deal with heat? **Cellular and Molecular Life Sciences**, [*s. l.*], v. 58, n. 9, p. 1216–1233, 2001.

LI, J. Mechanism of salt-induced activity enhancement of a marine-derived laccase, Lac15. **European Biophysics Journal**, [*s. l.*], v. 47, n. 3, p. 225–236, 2018.

LIU, J. Scalable production, fast purification, and spray drying of native Pycnoporus laccase and circular dichroism characterization. **Journal of Cleaner Production**, [*s. l.*], v. 127, p. 600–609, 2016.

LIU, Y. HUANG, L.; GUO, W.; JIA, L.; FU, Y.; GUI, S.; LU, F. Cloning, expression, and characterization of a thermostable and pH-stable laccase from *Klebsiella pneumoniae* and its application to dye decolorization. **Process Biochemistry**, [*s. l.*], v. 53, p. 125–134, 2017.

LOBLEY, A.; WHITMORE, L.; WALLACE, B. A. DICHROWEB: an interactive website for the analysis of protein secondary structure from circular dichroism spectra. **Bioinformatics**, [*s. l.*], v. 18, n. 1, p. 211–212, 2002.

LÓPEZ-GALLEGO, F. Improved stabilization of chemically aminated enzymes via multipoint covalent attachment on glyoxyl supports. **Journal of Biotechnology**, [*s. l.*], v. 116, n. 1, p. 1–10, 2005.

LÓPEZ-GALLEGO, F. Improved stabilization of chemically aminated enzymes via multipoint covalent attachment on glyoxyl supports. **Journal of Biotechnology**, [*s. l.*], v. 116, n. 1, p. 1–10, 2005b.

MAINARDI, P. H. Laccase production in bioreactor scale under saline condition by the marine-derived basidiomycete Peniophora sp. CBMAI 1063. **Fungal Biology**, [*s. l.*], v. 122, n. 5, p. 302–309, 2018.

MATEO, C.; ABIAN, O.; FERNANDEZ-LAFUENTE, R.; GUISAN, J. M. Reversible enzyme immobilization via a very strong and nondistorting ionic adsorption on support-polyethylenimine composites. **Biotechnology and Bioengineering**, [*s. l.*], v. 68, n. 1, p. 98–105, 2000.

MATYASH, L. F.; OGLOBLINA, O. G.; STEPANOV, V. M. Modification of carboxyl groups in pepsin. **European Journal of Biochemistry**, [*s. l.*], v. 35, n. 3, p. 540–545, 1973.

MAYER, A. M; STAPLES, R. C. Laccase: new functions for an old enzyme. **Phytochemistry**, [*s. l.*], v. 60, n. 6, p. 551–565, 2002.

MONTES, T. Chemical Modification of Protein Surfaces To Improve Their Reversible Enzyme Immobilization on Ionic Exchangers. **Biomacromolecules**, [*s. l.*], v. 7, n. 11, p. 3052–3058, 2006.

MUKHOPADHYAY, A.; DASGUPTA, A. K.; CHAKRABARTI, K. Enhanced functionality and stabilization of a cold active laccase using nanotechnology based activation-immobilization. **Bioresource Technology**, [*s. l.*], v. 179, p. 573–584, 2015.

MUKHOPADHYAY, M.; BANERJEE, R. Purification and biochemical characterization of a newly produced yellow laccase from Lentinus squarrosulus MR13. **3 Biotech**, [*s. l.*], v. 5, n. 3, p. 227–236, 2015.

MUTHUVELU, K. S.; RAJARATHINAM, R.; SELVARAJ, R. N.; RAJENDREN, V. B. A novel method for improving laccase activity by immobilization onto copper ferrite nanoparticles for lignin degradation. **International Journal of Biological Macromolecules**, [*s. l.*], v. 152, p. 1098–1107, 2020.

NAKAJIMA, N.; IKADA, Y. Mechanism of amide formation by carbodiimide for bioconjugation in aqueous media. **Bioconjugate Chemistry**, [*s. l.*], v. 6, n. 1, p. 123–130, 1995.

PERFETTI, R. B.; ANDERSON, C. D.; HALL, P. L. The chemical modification of papain with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. **Biochemistry**, [*s. l.*], v. 15, n. 8, p. 1735–1743, 1976.

PESSELA, B. C. C.; MUNILLA, R.; BETANCOR, L.; FUENTES, M.; CARRASCOSA, A. V.; VIAN, A.; FERNANDEZ-LAFUENTE, R.; GUISÁN, J. M. Ion exchange using poorly activated supports, an easy way for purification of large proteins. **Journal of Chromatography A**, [*s. l.*], v. 1034, n. 1–2, p. 155–159, 2004.

PESSELA, B. C. C.; FILHO, M.; MATEO, C.; CARRASCOSA, A. V.; FERNANDEZ-LAFUENTE, R.; GUISÁN, J. M. Purification and very strong reversible immobilization of large proteins on anionic exchangers by controlling the support and the immobilization conditions. **Enzyme and Microbial Technology**, [*s. l.*], v. 39, n. 4, p. 909–915, 2006.

PEZZELLA, C.; GUARINO, L.; PISCITELLI, A. How to enjoy laccases. **Cellular and Molecular Life Sciences**, [*s. l.*], v. 72, n. 5, p. 923–940, 2015.

PISZKIEWICZ, S.; PIELAK, G. J. Protecting enzymes from stress-induced inactivation. review-article. **Biochemistry**, [*s. l.*], v. 58, n. 37, p. 3825–3833, 2019.

QIU, X. Laccase immobilized on magnetic nanoparticles modified by amino-functionalized ionic liquid via dialdehyde starch for phenolic compounds biodegradation. **Chemical Engineering Journal**, [*s. l.*], v. 391, 2019, p. 123564, 2020.

QUE, Y. High-level coproduction, purification and characterisation of laccase and exopolysaccharides by Coriolus versicolor. **Food Chemistry**, [*s. l.*], v. 159, p. 208–213, 2014.

REIS, C. L. B.; DE SOUSA, E. Y. A.; DE FRANÇA SERPA, J.; OLIVEIRA, R. C.; DOS SANTOS, J. C. S. **Design of immobilized enzyme biocatalysts: Drawbacks and opportunities**. [*s. l.*], 2019.

RODRIGUES, R. C. Amination of enzymes to improve biocatalyst performance: coupling genetic modification and physicochemical tools. **RSC Adv.**, [*s. l.*], v. 4, n. 72, p. 38350–38374, 2014.

RODRIGUES, R. C. Immobilization-stabilization of the lipase from Thermomyces lanuginosus: Critical role of chemical amination. **Process Biochemistry**, [*s. l.*], v. 44, n. 9, p. 963–968, 2009.

RODRIGUES, R. C. Modifying enzyme activity and selectivity by immobilization. **Chem. Soc. Rev.**, [*s. l.*], v. 42, n. 15, p. 6290–6307, 2013.

RODRÍGUEZ COUTO, S.; TOCA HERRERA, J. L. **Industrial and biotechnological applications of laccases: A review**. [*s. l.*], 2006.

ROTH, S.; SPIESS, A. C. **Laccases for biorefinery applications: a critical review on challenges and perspectives**, [*s. l.*], 2015.

RUEDA, N.; DOS SANTOS, J. C. S.; ORTIZ, C.; TORRES, R.; BARBOSA, O.; RODRIGUES, R. C.; BERENGUER-MURCIA, Á.; FERNANDEZ-LAFUENTE, R. Chemical modification in the design of immobilized enzyme biocatalysts: Drawbacks and opportunities. **The Chemical Record**, [*s. l.*], v. 16, n. 3, p. 1436–1455, 2016.

SANTOS, J. C. S.; BARBOSA, O.; ORTIZ, C.; BERENGUER-MURCIA, A.; RODRIGUES, R. C.; FERNANDEZ-LAFUENTE, R. Importance of the Support Properties for Immobilization or Purification of Enzymes. **ChemCatChem**, [*s. l.*], v. 7, n. 16, p. 2413–2432, 2015.

SCHNEIDER, P. Characterization of a *Coprinus cinereus* laccase. **Enzyme and Microbial Technology**, [*s. l.*], v. 25, n. 6, p. 502–508, 1999.

SHELDON, R. A.; WOODLEY, J. M. Role of biocatalysis in sustainable chemistry. **Chemical Reviews**, [*s. l.*], v. 118, n. 2, p. 801–838, 2018.

SILVA, J. A.; MACEDO, G. P.; RODRIGUES, D. S.; GIORDANO, R. L. C.; GONÇALVES, L. R. B. Immobilization of *Candida antarctica* lipase B by covalent attachment on chitosan-based hydrogels using different support activation strategies. **Biochemical Engineering Journal**, [*s. l.*], v. 60, p. 16–24, 2012.

STERNER, R.; LIEBL, W. Thermophilic adaptation of proteins. **Critical Reviews in Biochemistry and Molecular Biology**, [*s. l.*], v. 36, n. 1, p. 39–106, 2001.

SU, J. Laccase: a green catalyst for the biosynthesis of poly-phenols. **Critical Reviews in Biotechnology**, [*s. l.*], v. 38, n. 2, p. 294–307, 2018.

UPADHYAY, P.; SHRIVASTAVA, R.; AGRAWAL, P. K. Bioprospecting and biotechnological applications of fungal laccase. **3 Biotech**, [*s. l.*], v. 6, n. 1, p. 15, 2016.

VIEIRA, M. F. β-Glucosidase immobilized and stabilized on agarose matrix functionalized with distinct reactive groups. **Journal of Molecular Catalysis B: Enzymatic**, [*s. l.*], v. 69, n. 1–2, p. 47–53, 2011.

WHITMORE, L.; WALLACE, B. A. DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. **Nucleic Acids Research**, [*s. l.*], v. 32, p. W668–W673, 2004.

WHITMORE, L.; WALLACE, B. A. Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases. **Biopolymers**, [*s. l.*], v. 89, n. 5, p. 392–400, 2008.

WOODY, R. W. **Circular dichroism**, [*s. l.*], v. 11, p. 34–71, 1995.

XU, B.; ZHAN, G.; XU, B.; DU, H.; LUO, H.; WANG, T.; ZHAN, C.; YANG, Y. Degradation of acetaminophen in aqueous solution by UV and UV-activated sludge processes. **Water Science and Technology**, [*s. l.*], v. 78, n. 10, p. 2088–2095, 2018.

ZUCCA, P.; FERNANDEZ-LAFUENTE, R.; SANJUST, E. Agarose and its derivatives as supports for enzyme immobilization. **Molecules**, [*s. l.*], v. 21, n. 11, p. 1577, 2016.

Chapter 4

Immobilization of laccase multilayers in porous support as a strategy to increase biocatalyst performance

4.1 Abstract

This work proposes the obtainment of a two-layer laccase biocatalyst using genipin as the main crosslinker. The multilayered biocatalysts were prepared following different combinations of genipin and glutaraldehyde in the individual preparation of the first and of the second laccase layers. First, chitosan was treated with genipin or glutaraldehyde, followed by the immobilization of the first laccase layer to form a single-layer biocatalyst. Then, the immobilized laccases were coated once again with genipin or glutaraldehyde, and a new laccase layer would be immobilized onto the system, giving the final multi-layer biocatalyst. An increase in catalytic activity in the orders of 1.7- and 3.4-fold was achieved when glutaraldehyde coating was used to prepare the second laccase layer, compared to the single-layer biocatalysts. However, the addition of a second layer did not always produce more active biocatalysts, since the two-layer biocatalysts prepared with genipin coating presented a decrease in activity when compared to their single-layer counterparts. The stability values for all the double-layer biocatalysts were lower than those observed in the single-layer systems. Nevertheless, the double-layer, genipin-coated biocatalyst promoted a higher removal of trace organic contaminants, since it reached removal levels of 100% of mefenamic acid, and of 66% of acetaminophen, compared with glutaraldehyde-coated biocatalyst that degraded 20% of mefenamic acid, and of 18% of acetaminophen.

Keywords: Chitosan. Genipin. Glutaraldehyde. Laccase. Layer-by-Layer. Pollutant Removal.

4.2 Introduction

The current research carried out on bioprocess engineering topics has heavily focused on the design of green and environmentally sustainable biocatalysts (VIRGEN-ORTÍZ et al., 2017b). In order to achieve this, new enzymatic biocatalysts have been studied and developed over the last years (WOODLEY, 2008). Among them, laccase-based biocatalysts have gained attention due to their great biotechnological potential (DARONCH et al., 2020).

Laccases (EC. 1.10.3.2) are glycoproteins widely used in the degradation of micropollutants from water due to their capability to catalyze a wide variety of phenolic substrates (RODRÍGUEZ COUTO; TOCA HERRERA, 2006) with concomitant reduction of oxygen to water. Nevertheless, inherent issues such as loss of stability, high production costs, and water solubility can limit laccase applications (BRUGNARI et al., 2018). Thus, byocatalist immobilization is an important step for overcoming these limitations, since this process can optimize the native characteristics of the biocatalyst and, consenquently, its applicability (BEZERRA et al., 2015). If well designed and implemented, immobilization strategies can provide the reaction systems with many interesting advantages, such as increased enzymatic activity and stability, better control in product formation, as well as improved process efficiency, selectivity, and enzyme recovery (SANTOS et al., 2015).

Layer-by-layer immobilization is a method that focus on the preparation of solid biocatalysts by alternating layers of enzyme and bonding materials in order to create a stack of enzyme layers (ARANA-PEÑA; RIOS; MENDEZ-SANCHEZ; LOKHA; GONÇALVES; et al., 2020; FERNÁNDEZ-FERNÁNDEZ; SANROMÁN; MOLDES, 2013). These multilayers can be formed by electrostatic or hydrophobic interactions, hydrogen or covalent bonding, or by a combination of these (NGUYEN, H. H.; KIM, 2017; ZHANG, J. et al., 2020). The strategy is believed to enhance the loading capacity of the support (ARANA-PEÑA; RIOS; MENDEZ-SANCHEZ; LOKHA; GONÇALVES; et al., 2020) by the addition of an increased amount of the desired enzyme, consequently improving the global activity of the biocatalyst.

In this work, we propose a strategy based on a layer-by-layer immobilization of laccase in order to produce a highly active biocatalyst. To this end, genipin was chosen as the main crosslinker due to the lower toxicity presented relative to others, such as glutaraldehyde (MANICKAM; SREEDHARAN; ELUMALAI, 2014). Glutaraldehyde was also tested in the production of laccase multilayers since it is a bifunctional agent that has been widely used

over the last years to immobilize enzymes owing to its versatility (BARBOSA et al., 2014; PINHEIRO et al., 2019). However, due to its native toxicity and poor stability under alkaline conditions (PINHEIRO et al., 2019), alternative strategies to produce new heterofuncional supports have been developed. Genipin, for example, emerges as an important replacement for glutaraldehyde due to its slower degradation rates, lower toxicity, and improved biocompatibility compared to materials crosslinked by glutaraldehyde (MANICKAM; SREEDHARAN; ELUMALAI, 2014). Genipin is a hydrolytic product of geniposide that shows high stability and biocompatibility (BELLÉ et al., 2018; CUI et al., 2017). As a bifunctional compound, it can react with the support and the proteins in the medium (MANICKAM; SREEDHARAN; ELUMALAI, 2014; MUZZARELLI et al., 2016) via covalent, ion exchange, or hydrophobic interactions (TACIAS-PASCACIO et al., 2019).

It has been shown that the characteristics of the support used in the immobilization of enzymes greatly influence the performance of the biocatalyst (SHELDON; VAN PELT, 2013). Chitosan is a natural, low-cost, non-toxic, and biocompatible polymer with chemical resistance, relative thermal stability, and convenient mechanical properties (AVCU et al., 2019). As support for enzyme immobilization, it is a versatile polymer due to its active amino groups that act as a reactive site for new groups (ALI; AHMED, 2018). Moreover, chemical modification is required if the enzyme is to be covalently immobilized onto the support (MENDES et al., 2013b). Chitosan activation with genipin or glutaraldehyde provides a heterofuncional support (BARBOSA et al., 2013). The reagents react with the amino groups in the chitosan by forming covalent bonds, and they show a strong pH dependency that is linked to the degree of chitosan protonation (DELMAR; BIANCO-PELED, 2015).

Laccase-based biocatalysts has been used as an alternative for the remove of micropollutants from water. Over the past decades**,** the unprecedented population increase has caused, among other consequences, the worsening of the water pollution issue, which affect the health of both humans and aquatic organisms (MORSI et al., 2020). Most of the pharmaceutical compounds found in wastewaters are composed of drug molecules that are frequently prescribed or purchased over the counter, such as acetaminophen and mefenamic acid (BA et al., 2014; MOLL et al., 2011; XU, B. et al., 2018). The molecular structure of acetaminophen includes a phenol ring, which renders this compound stable and difficult to degrade (XU, B. et al., 2018). The mefenamic acid molecule, in turn, possesses amine, carboxylic, and benzylic rings, and also methyl groups that present low aqueous solubility and high permeability through biological membranes (MUDALIP et al., 2016).

Thus being, this research aimed to produce two-layer biocatalysts using laccases previously immobilized onto chitosan. Genipin and glutaraldehyde were used as cross-linking agents. The immobilization parameters and stability of the final biocatalysts were determined, and their potential for the removal of the aforementioned trace organic contaminants from aqueous solutions was also assessed.

4.3 Materials and methods

4.3.1 Materials

2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), glutaraldehyde (25% w/w in water), 1-ethyl-3-(3-dimethylamino propyl) carbodiimide (EDAC), genipin, and laccase from Aspergillus sp (Novozymes 51003, EC 420.150.4) were purchased from MilliporeSigma (Oakville, ON, Canada). Low-molecular-wight chitosan was purchased from Fluka BioChemika. All the reagents used were of analytical grade or higher.

4.3.2 Methods

4.3.2.1 Support characterization

4.3.2.1.1 Elemental Analysis

Elemental analyses to determine the carbon, nitrogen, and oxygen levels of the pure and the activated chitosan were performed (Truspec Micro, with the Micro O module). The oven temperature for the analyses of carbon (C) and nitrogen (N) was 1050 °C, while for the analysis of oxygen (O), it was 1350 °C.

4.3.2.1.2 Scanning electron microscopy (SEM)

A SEM characterization (SEM FEG Quanta 450 with Energy Dispersive X-Ray Spectroscopy (EDS)) of the surface of the supports was performed under an incident electron beam of 20 kV. The samples were deposited on carbon tape and metalized with gold by the Metalizator Quorum QT150ES.

4.3.2.2 Chitosan activation with genipin and glutaraldehyde

The biocatalyst production started with the activation of the low-molecular-weight chitosan with one of the crosslinkers (glutaraldehyde or genipin) using a ratio of of 7.5% ($w_{crosslinker}$:$w_{chitosan}$). Chitosan activation with genipin was performed by first dissolving 0.05 g of chitosan in 2 mL of a 10% (v/v) acetic acid solution (adjusted with NaOH 2M to pH 5.0) (MARIA et al., 2014). The system was maintained under agitation at 100 rpm for 30 min, followed by the addition of 0.375 mL of a 1% (m/v) genipin solution (0.00375 g of genipin) to achieve a final concentration of 7.5% ($w_{genipin}$/$w_{chitosan}$) (0.007 M). The 1% (m/v) genipin solution was prepared dissolving 0.01 g of genipin in 1 mL of 10% (v/v) acetic acid (adjusted with NaOH 2M to pH 5.0) (DELMAR; BIANCO-PELED, 2015). The system was left under mild agitation (100 rpm) for 6 h. Finally, the solution was added dropwise to a 2M NaOH solution by using a syringe (10 mL) and left under agitation at 60 rpm for 1h a 25 °C. Subsequently, the chitosan beads were washed with distilled water until neutrality was reached.

Similarly, the chitosan activation with glutaraldehyde was performed to achieve a final concentration of 7.5% ($w_{glutaraldehyde}$:$w_{chitosan}$) (0.075 M). Initially, 0.05 g of chitosan was added to 2 mL of acetic acid solution 10% (v/v) pH 5.0 for 30 min under agitation at 100 rpm (CHAGAS et al., 2015), followed by dripping the solution into 2M NaOH with a syringe (10 mL) and agitation at 60 rpm. Then, the chitosan beads were washed with distilled water to neutrality. 1.0 g of dry chitosan beads was added to 9.717 mL of 0.1 M sodium phosphate buffer at pH 7.0 and 0.283 mL of glutaraldehyde 25% (v/v) (0.075 g of glutaraldehyde), in order to achieve a final concentration of 7.5 % ($w_{glutaraldehyde}$:$w_{chitosan}$). The system was kept under constant agitation for 1 h at room temperature (SILVA et al., 2012). Afterwards, the support was washed with distilled water until neutrality was reached.

4.3.2.3 Laccase Immobilization

4.3.2.3.1 First Laccase Layer

Laccase immobilization was carried out using 10 U of commercial laccase per gram (1.5 mg of protein/g) of pre-activated chitosan in 10 mM sodium acetate buffer at pH 5.0 containing 1.2 mM of EDAC (NAGHDI et al., 2018a), for 16 hours.

4.3.2.3.2 Glutaraldehyde/genipin coatings

An amount of 1.0 g of each biocatalyst (GenLac and GluLac) was treated separately with 10 mL of glutaraldehyde solution 1% (v/v) in 50 mM of sodium phosphate buffer at pH 7.0 for 1 h (2.66 % $w_{glutaraldehyde}$:$w_{biocatalyst}$) (ARANA-PEÑA; RIOS; MENDEZ-SANCHEZ; LOKHA; GONÇALVES; et al., 2020). These conditions are known to allow for the full modification of the primary amino groups of proteins by one molecule of glutaraldehyde (ARANA-PEÑA; RIOS; MENDEZ-SANCHEZ; LOKHA; GONÇALVES; et al., 2020). Thus being, the coated biocatalysts GenLacGlu and GluLacGlu were prepared. Finally, the biocatalysts were filtered and washed with distilled water.

Similarly, the genipin coating process was performed using the same mass ratio of 2.66 % $w_{genipin}$:$w_{biocatalyst}$ in 10 mM sodium acetate buffer at pH 5.0 for 6h. With this process, the coated biocatalysts GenLacGen and GluLacGen were obtained.

4.3.2.3.3 Second Laccase layer

The same protocol used for the immobilization of the first laccase layer was followed for the second (see section 2.2.3.1). 10 U of commercial laccase was added to 1.0 g of the coated biocatalysts (GenLacGlu, GluLacGlu, GenLacGen, or GluLacGen) in 10 mM of sodium acetate buffer at pH 5.0 and 1.2 mM of EDAC for 16 hours. The final biocatalysts were: GenLacGluLac, GluLacGluLac, GenLacGenLac, and GluLacGenLac.

4.3.2.4 Enzyme activity and protein concentration assay

Laccase activity was determined by monitoring the oxidation of ABTS to its radical cation ($ABTS^{\cdot+}$) at 420 nm (molar extinction coefficient ε_{ABTS} = 36,000 M^{-1} cm^{-1}) (ADDORISIO et al., 2013) in a microliter plate reader. The activity was measured using 10 mL of enzyme solution, 100 mL of sodium acetate buffer 10 mM pH 4.5, and 90 mL of 1 mM ABTS in 10 mM sodium acetate buffer pH 4.5. The duration of the reaction was 60 s.

The oxidation of ABTS using chitosan beads was performed in a beaker. A mass of biocatalyst was added to 3.3 mL of sodium acetate buffer 10 mM pH 4.5 and 2.7 mL of 1 mM ABTS in 10 mM of sodium acetate buffer pH 4.5. Samples (200 mL) were withdrawn every minute for five minutes, and the absorbance was measured using a microliter plate

reader. One international unit (IU) of laccase activity corresponds to the oxidation of 1 mmol ABTS per minute under these conditions.

The bicinchoninic acid (BCA) test kit from Thermo Fisher Scientific (Rockford, Illinois, United States) was used to determine the total protein content at 562 nm. Bovine serum albumin was used as a standard (SMITH et al., 1985).

4.3.2.5 Biocatalyst characterization

4.3.2.5.1 Immobilization parameters

The immobilization yield (IY, %), theoretical activity (At_t, U/g), and recovery activity (At_r, %) of the biocatalysts were calculated according to Silva et al. (2012) (SILVA et al., 2012). The immobilization yield (IY, %) was calculated as the difference between the initial activity (At_i) and the remaining enzyme activity in the supernatant (At_f), divided by the initial activity. This parameter describes the percentage of enzyme immobilized onto the support, according to Eq. (1).

$$IY(\%) = \frac{At_i - At_f}{At_i} x100$$

(1)

The recovered activity (At_r, %), in turn, is the biocatalyst activity (At_d, U/g) divided by the theoretical activity (At_t, U/g) of the laccase immobilized onto the support, i.e., the amount of laccase offered per gram of support multiplied by the immobilization yield, according to Eq. (2).

$$At_r(\%) = \frac{At_d}{At_t} x100$$

(2)

A statistical comparison between the parameters of the different biocatalysts was performed via an analysis of variance (ANOVA). All the statistical analyses were carried out using Excel (version 16.45), considering significant differences at $p < 0.05$.

4.3.2.5.2 Thermal and Operational stabilities

The thermal inactivation of the different biocatalysts was investigated by incubating a given mass of the biocatalysts in 10 mM of sodium acetate buffer pH 4.0 at 40 °C for 4h, 8h, 16h, and 24h. The laccase activity in both solid biocatalysts, as well as in the supernatant, was measured as previously described (see section 2.2.4.).

The operational stability (reuse) of the solid biocatalysts was evaluated by the oxidation of 1 mM ABTS in 10 mM of sodium acetate buffer pH 4.5 with a total volume of 5 mL. Consecutive cycles of ABTS oxidation reactions were performed with the same biocatalyst. After each cycle, the solution was removed with a pipette and the biocatalyst was washed with approximately 10 mL of 10 mM sodium acetate buffer pH 4.5. The relative activity of the biocatalyst was calculated in each cycle, assuming an activity in the first cycle of 100%.

4.3.2.5.3 Enzymatic removal of trace organic contaminants

Lastly, the elimination of the non-steroidal anti-inflammatory (NSAI) drugs acetaminophen and mefenamic acid was used as a model reaction to determine the potential of the use of these biocatalysts for bioremediation purposes. The catalysts were added to solutions of each of the NSAI drugs to obtain final activities of 25 U/L. The experiments were performed at pH 7.0, 25 °C, 135 rpm, and for 24 h. The final concentration of the target compound was 100 ng/mL. After 24 hours, an aliquot of the supernatant was taken to measure the activity by spectrophotometry, and the degradation level was analyzed by UPLC/MS-MS (HAROUNE et al., 2014). The analyses of acetaminophen and mefenamic acid were performed by liquid chromatography coupled to a mass spectrometer (Waters Xevo TQ MS) equipped with an Acquity UPLC HSS-T3 column (100 mm x 2.1, 1.8 μm) and a 0.2 μm pre-filter (HAROUNE et al., 2014). The solvent flow rate was set to 0.4 mL/min. The mobile phase was 0.1% formic acid/water (A) and 0.1% formic acid/methanol-acetonitrile (80-20 v/v) (B). All the experiments were performed in triplicate and the results are expressed as their average and standard deviations.

4.4 Results and discussion

4.4.1 Characterization of modified chitosan

4.4.1.1 Elemental analysis and scanning electron microscopy (SEM)

In order to characterize the surface of the modified chitosan, an elemental analysis was performed. The results are summarized in Table 6.

Table 6 - Elemental analysis (carbon, nitrogen, oxygen) of the non-modified chitosan and of the chitosan activated with genipin and glutaraldehyde

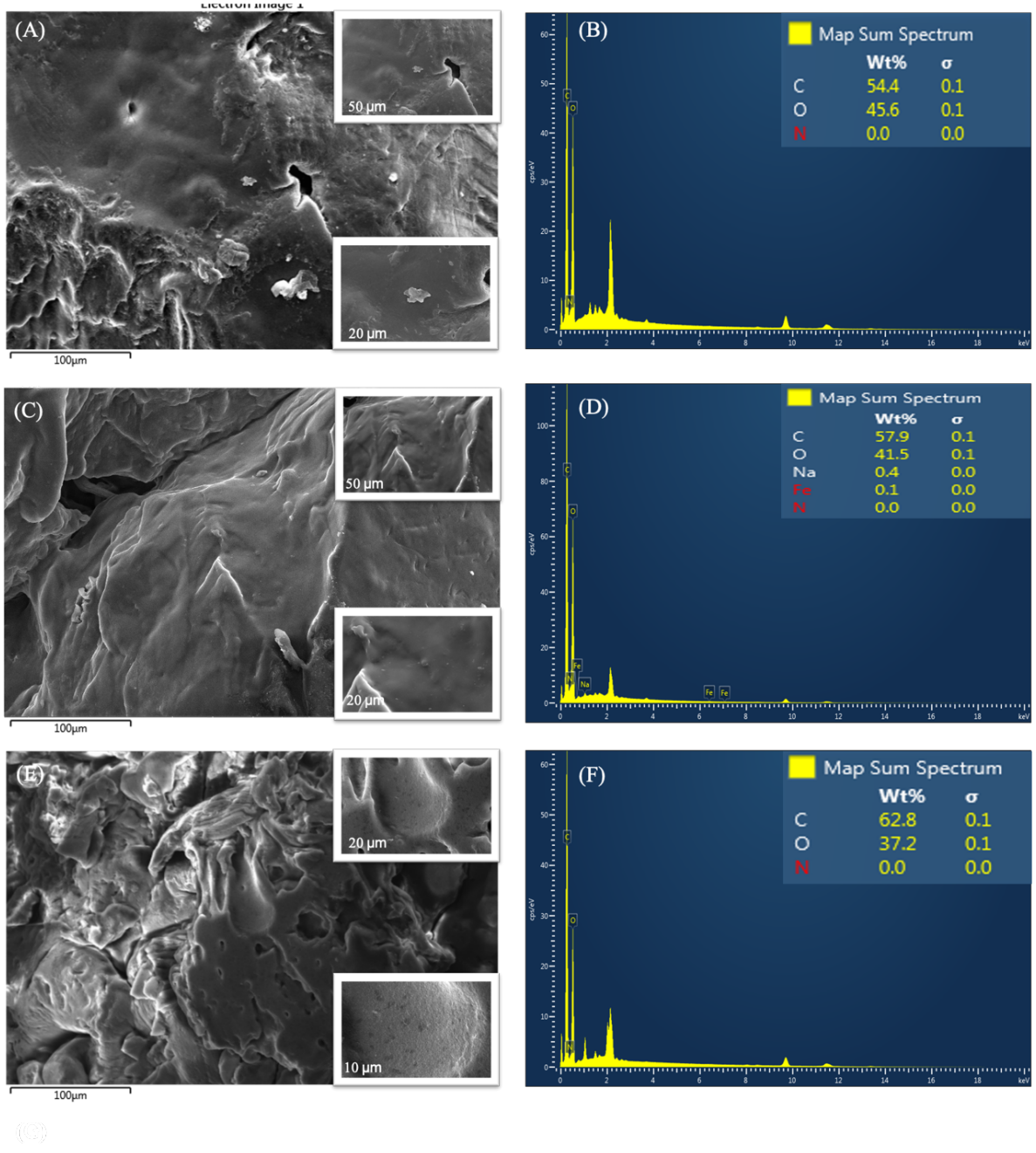
Materials	%C	%N	%O
Non-modified Chitosan	40.5	7.6	45.0
Chitosan activated with genipin	41.1	7.3	42.3
Chitosan activated with glutaraldehyde	42.8	4.7	38.2

Source: elaborated by the author.

The results show a decrease in the percentages of nitrogen and oxygen, and an increase in the carbon percentage of the modified chitosan compared to the non-modified material. The decrease observed in the nitrogen percentage indicates an increase in the number of groups added to the support owing to the reaction of glutaraldehyde and genipin with the amino groups of chitosan (KILDEEVA et al., 2009). On the other hand, the nitrogen content remained fairly constant after the activation with genipin. However, it is important to mention that chitosan beads underwent a color change from pearly white to dark blue after reacting with genipin due to an oxygen radical-induced polymerization of the crosslinker (TAVARES et al., 2020).

The SEM images, EDS spectra, and EDS maps of the samples with their composition information are shown in Figure 13. The surface of the pure chitosan (Figure 13A) appears to be rough and presents low porosity. Figures 13C and 13E suggest an increase in the native roughness and porosity of chitosan following its activation with genipin and glutaraldehyde, respectively. Activation with genipin provoked the creation of multiple porous compartments and a higher roughness, a fact that was also observed by Tavares et al. (2020) (TAVARES et al., 2020). EDS maps were collected for all the samples (100 μm) to qualitatively evaluate the dispersion of the elements in the surface supports. As expected, an increase in carbon content was observed for the samples activated with genipin (Figure 13D) and glutaraldehyde (Figure 13F), compared to that of pure chitosan (Figure 13B). For instance, the carbon percentage increased from 54.4 in the pure chitosan to 57.9 genipin-activated chitosan.

Figure 13 - SEM-images, EDS-maps and EDS-spectra of pure chitosan (A and B); chitosan activated with genipin 7.5 % (w/w) (C and D); and chitosan activated with glutaraldehyde 7.5 % (w/w) (E and F)



Source: elaborated by the author.

4.4.2 Biocatalysts parameters

As described in section *4.3.2.3*, laccase immobilization in the activated supports was carried out offering 10 U/g. The immobilization parameters are presented in Table 7.

Table 7 - Immobilization yield (IY), biocatalyst activity (At_D), and recovery activity of the biocatalysts: chitosan-immobilized laccase activated with genipin (GenLac), chitosan-immobilized laccase activated with glutaraldehyde (GluLac); second layer of laccase immobilized in glutaraldehyde-coated GenLac (GenLacGluLac), second layer of laccase immobilized in glutaraldehyde-coated GluLac (GluLacGluLac), second layer of laccase immobilized in genipin-coated GenLac (GenLacGenLac), second layer of laccase immobilized in genipin-coated GluLac (GluLacGenLac)

Biocatalyst[β]	**IY (%)**	**Biocatalyst Activity (U/g)**	**Recovery Activity (%)**
GenLac	98.6 ± 0.1	2.0 ± 0.2	20.1 ± 1.8
GluLac	98.2 ± 0.0	1.4 ± 0.1	14.2 ± 0.7
GenLacGluLac	99.3 ± 0.1	3.4 ± 0.1	33.8 ± 0.9
GluLacGluLac	91.1 ± 0.3	4.8 ± 0.3	52.5 ± 2.9
GenLacGenLac	98.8 ± 0.0	0.7 ± 0.1	7.3 ± 0.6
GluLacGenLac	97.2 ± 0.0	1.0 ± 0.1	10.1 ± 1.1

Source: elaborated by the author.

According to Table 7, the single-layer laccase biocatalysts GenLac and GluLac presented similar immobilization yields of around 98.6% and 98.2%, respectively. However, the activity of GenLac (2.0 U/g) was higher than that of GluLac (1.4 U/g). This behavior can be attributed to some factors. One possible explanation for this is related to the laccase conformation adopted during the immobilization reaction. The immobilization mechanism using glutaraldehyde involves a predominant reaction with the amino terminal group (KLEIN et al., 2016) and other amino groups of lysine (TOMIMATSU et al., 1971) in the enzyme; genipin, on the other hand, is more susceptible to crosslinking with amino groups of lysine or arginine residues (SUNG et al., 2001). Thus, it is expected that the use of the different crosslinking agents results in the manufacture of biocatalysts with distinct properties since the different reactivities can provide unique orientations for the laccase in each biocatalyst.

The lower activity levels observed in GluLac compared to GenLac could be explained by enzyme rigidification, and the obstruction or damage of the laccase active site, by consequent conformational changes (BILAL et al., 2019; ZHENG et al., 2016). Also, the activities and recovery activities of both biocatalysts were low, which can be related to diffusion restrictions encountered by some enzymes immobilized onto porous supports, as is the case with chitosan. This impedes the substrate from accessing the reaction site, thus decreasing the immobilization efficiency (BORTONE; FIDALEO; MORESI, 2014).

Moreover, some distortion of the enzyme structure during the immobilization may occur once a multipoint covalent bond is formed (MATEO, CÉSAR et al., 2006). Also, neither the support nor the functional groups are inert, as they exert an electrostatic field that modifies all the electrostatic interactions taking place in the vicinity of the surface, altering the behavior of buffer salts, substrates, products, and of the enzyme itself (HOARAU; BADIEYAN; MARSH, 2017). Therefore, the activated support is believed to affect the physicochemical properties of the neighboring molecules. Although support activation usually limit surface effects, it can still play a key role on enzyme activity (HOARAU; BADIEYAN; MARSH, 2017).

The two-layer laccase biocatalysts prepared via a glutaraldehyde coating of the single-layer biocatalysts showed a moderate increase in activity. GenLacGluLac presented an activity 1.7-fold higher than that of GenLac, while GluLacGluLac, presented one that was 3.4-fold higher than that of GluLac (Table 7). The considerable difference between the GluLacGluLac and GenLacGluLac activities may be related to the impact of the laccase bonds in each layer, once the same crosslinker was used to prepare the first and second layers. The second enzyme layer may adopt a better conformation when immobilized onto glutaraldehyde, once these groups leave a space large enough for the substrate to reach the active center of laccase (BARBOSA et al., 2013; RIOS et al., 2019). Furthermore, the glutaraldehyde treatment can also strengthen the interaction between the laccase and the previous layer of crosslinking agent, and induce an improvement in the biocatalyst activity after the immobilization of the second layer, since this leads to an increase in the amount of immobilized enzyme (CHRISTWARDANA, 2017) (Table 7). The work of Semerdzhieva et al. (2018) (SEMERDZHIEVA V, RAYKOVA R, MARINKOVA D, YANEVA S, 2018) seems to corroborate this hypothesis. The authors investigated a layer-by-layer immobilization of laccase onto Whatman 1 paper as a support and with the crosslinking agents polyethyleneimine (PEI) and glutaraldehyde. These authors also reported an increase in biocatalyst activity after the glutaraldehyde treatment compared to the single layer of laccase.

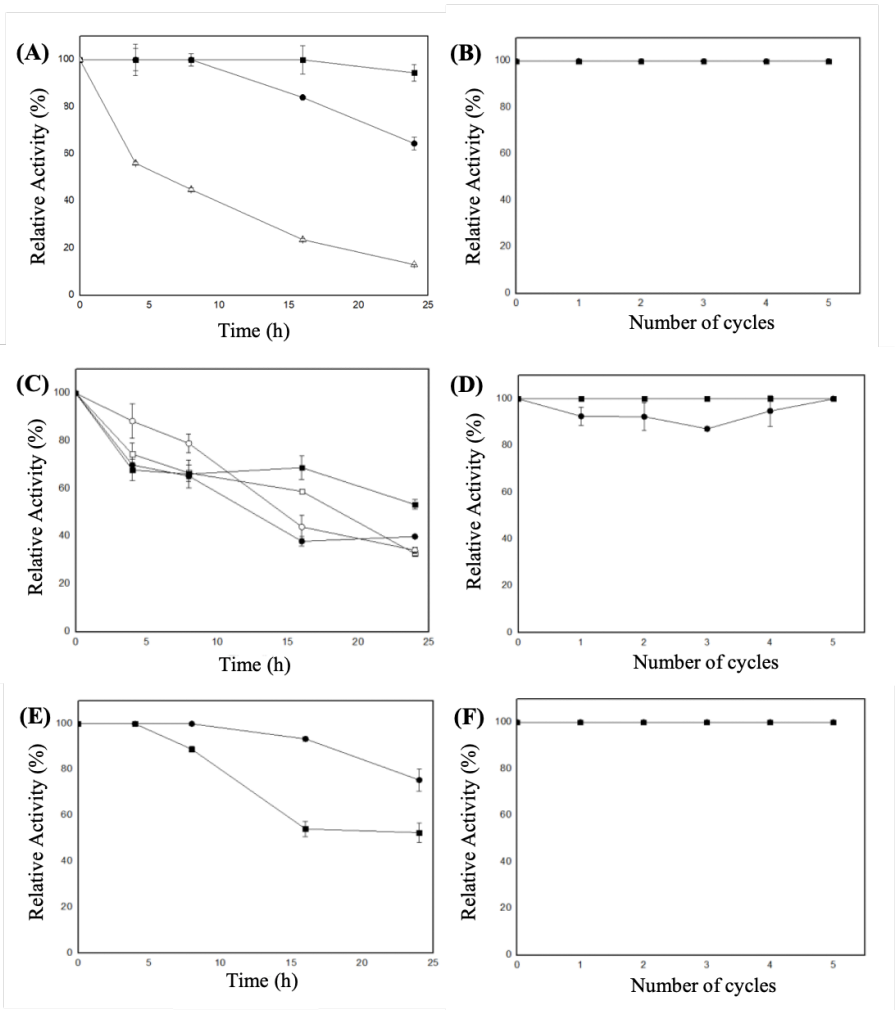
The coating of the single-layer biocatalysts (GenLac and GluLac) with genipin (GenLacGen and GluLacGen) promoted a considerable decline in the apparent activity of laccase (results not shown). The network formed by genipin may have caused an increase in tortuosity, preventing the substrate (ABTS) from reaching the active sites of the first laccase layer (CRESTINI; PERAZZINI; SALADINO, 2010). Despite the high immobilization yield obtained, the double-layer biocatalysts (GenLacGenLac and GluLacGenLac) presented a

lower activity than their single-layer counterparts (GenLac and GluLac), which might be owing to the direct effect of the properties of the immobilized enzymes triggered by the coating process (ARANA-PEÑA; RIOS; MENDEZ-SANCHEZ; LOKHA; GONÇALVES; et al., 2020). Thus, this conformation was not shown to be favorable to the catalysis of the oxidation of ABTS. Also, the literature reports that genipin possess native antioxidant activity (LEE; LEE; JEONG, 2009), since the molecule is a phenolic compound and can act as a free-radical scavenger. On this subject, Nunes et al. (2013) reported a slight increase in the antioxidant activity of chitosan activated with genipin. Oliveira et al. (2020), on the other hand, reported a decrease in the antioxidant activity of the materials covered with chitosan-genipin layers. Therefore, the possibility of ABTS radical scavenging by genipin layers cannot be discarded.

4.4.3 Thermal and operational stabilities

A crucial parameter in the characterization of biocatalysts for industrial applications is their thermal and operational stabilities. In order to confirm the suitability of an immobilization protocol, the stability limitations of the free enzyme must be assessed (BOUDRANT; WOODLEY; FERNANDEZ-LAFUENTE, 2020), as indicated in the results shown in Figure 14.

Figure 14 - The thermal stability (A) of the biocatalysts ■ GenLac (At_{d0} = 1.7 ± 0.2 U/g), GluLac (At_{d0} = 1.9 ± 0.1 U/g), and ∆free laccase (At_{d0} = 0.4 ± 0.0 U/mL) at 40 °C and pH 4.0. The operational stability (B) was monitored by conducting 5 ABTS-oxidation cycles at 25 ºC and pH 4.5. (C) shows the thermal stability of the biocatalysts: □ GenLac coated with glutaraldehyde (GenLacGlu, At_{d0} = 1.4 ± 0.4 U/g), and ○ GluLac coated with glutaraldehyde (GluLacGlu, At_{d0} = 2.4 ± 0.1 U/g); and of the two-layer biocatalysts: ■ GenLacGluLac (At_{d0} = 2.6 ± 0.6 U/g), and · GluLacGluLac (At_{d0} = 4.6 ± 0.0 U/g), at 40 °C and pH 4.0. (D) shows the operational stability of the biocatalysts: ■ GenLacGluLac and · GluLacGluLac, which was monitored by conducting 5 ABTS-oxidation cycles at 25 ºC and pH 4.5. The thermal stability of the two-layer biocatalysts is presented in (E): ■ GenLacGenLac (At_{d0} = 0.5 ± 0.1 U/g) and · GluLacGenLac (At_{d0} = 1.2 ± 0.1 U/g), at 40 °C and pH 4.0. The operational stability (F) of the biocatalysts: ■ GenLacGenLac and · GluLacGenLac monitored by conducting 5 ABTS-oxidation cycles at 25 ºC and pH 4.5



Source: elaborated by the author.

The immobilized biocatalysts presented higher stability than the free laccase under acidic pH (Figure 14A), demonstrating a maintenance of the enzyme activity and the enhancement of its stability by the immobilization method (MA et al., 2018). This can be explained by the natural establishment of multiple covalent bonds between the laccase and the crosslinking agent (genipin or glutaraldehyde) during the reaction. However, enzyme leakage was detected in the supernatant with time, indicating that some enzyme molecules might have been weakly adsorbed onto the support (results not shown). As shown by the elemental analysis (*section 4.4.1.1*), after chitosan activation with genipin or glutaraldehyde, a decrease in the nitrogen percentage was observed, indicating an increase in the number of groups added to the support upon glutaraldehyde and genipin reaction with the amino groups of chitosan (KILDEEVA et al., 2009). However, this modification was not complete, given that some amino groups of chitosan were still detected in the elemental analysis (Table 6). Thus, the possibility of ionic interactions between the chitosan and the anionic groups of proteins cannot be discarded, since at acidic or neutral pH values, chitosan molecules are positively charged (KILINÇ; ÖNAL; TELEFONCU, 2002; MA et al., 2018). This type of interaction can also restrict the free movement of laccase and consequently enhance biocatalyst stability (KILINÇ; ÖNAL; TELEFONCU, 2002).

The biocatalysts prepared with chitosan activated with genipin (GenLac) presented higher thermal stability than those prepared with glutaraldehyde (GluLac). In the literature, it is reported that there is the possibility of a tertiary amine structure being produced by the reaction of genipin with the free amino groups, which generates a structure more stable than Schiff's bases (WANG; JIANG; XIONG, 2019). At low pH values of activation, the chains of chitosan-genipin are short and closed (FLORES et al., 2019), which conveys more rigidity to the enzyme. Moreover, the support and its functional groups modify the electrostatic interactions taking place close to the surface (HOARAU; BADIEYAN; MARSH, 2017), which alters the behavior of buffer salts, substrates, products, and of the enzyme itself (HOARAU; BADIEYAN; MARSH, 2017). Thus being, these results suggest that the chitosan activated with genipin positively affects the physicochemical properties of the laccase-based biocatalysts and enhances the thermal tolerance of GenLac compared to GluLac (Figure 14A).

The coating of the immobilized enzymes with glutaraldehyde promoted different effects on the thermal stability of biocatalysts. This result was expected since immobilized enzymes can adopt different conformations depending on the specific group contributions from the underlying layer (CARUSO; SCHÜLER, 2000), which impacts enzyme rigidity and thermal deactivation resistance. Also, during the coating process, the reactivity of the

glutaraldehyde with the support cannot be discarded (BARBOSA et al., 2014). A decrease in the thermal stability of the biocatalysts GenLac and GluLac after glutaraldehyde coating was observed, which can also be related to a degree of reactivity of the glutaraldehyde with the support and with the inner layer of laccase. Both biocatalysts were already very stable previous to the glutaraldehyde coating process (Figure 14A), and this extra step may have affected the immobilization that had been well established. An improvement in thermal stability was observed when a second laccase layer was added (GenLacGluLac and GluLacGluLac) compared to the single-layer biocatalysts (GenLacGlu and GluLacGlu). However, the two-layer biocatalysts were less stable than their single-layer counterparts (Figures 14A and 14C). The first laccase layer was most likely immobilized inside chitosan pores, which preserved its active sites and conformation transitions under various environmental conditions (SECUNDO, 2013). In contrast, the second laccase layer was probably immobilized in a more exposed position, which granted higher biocatalyst activity but with the trade-off of a higher susceptibility to environmental changes.–

The biocatalyst GenLacGenLac (Figure 14E) presented a decrease in thermal stability compared to the single-layer biocatalyst GenLac. However, GluLacGenLac showed better thermal stability compared to GluLac (Figure 14A and 14E). This is because the first laccase layer in the latter system had been prepared with glutaraldehyde following by genipin coating and by the immobilization of the second laccase layer. The interactions between the first genipin layer and the second laccase layer positively affected the stability of these biocatalysts, giving them a more stable conformation despite the catalyst not being as active. GluLacGenLac maintained 75.5 ± 4.9 % of its initial activity after 24h of incubation. The good stability of this biocatalyst can be attributed to a higher rigidity and compact packing (SECUNDO, 2013) of the enzyme. Besides, the different conformation adopted by the first (glutaraldehyde) (KLEIN et al., 2016; TOMIMATSU et al., 1971) and second layers (genipin) (SUNG et al., 2001) could have also contributed to this improvement in system stability.

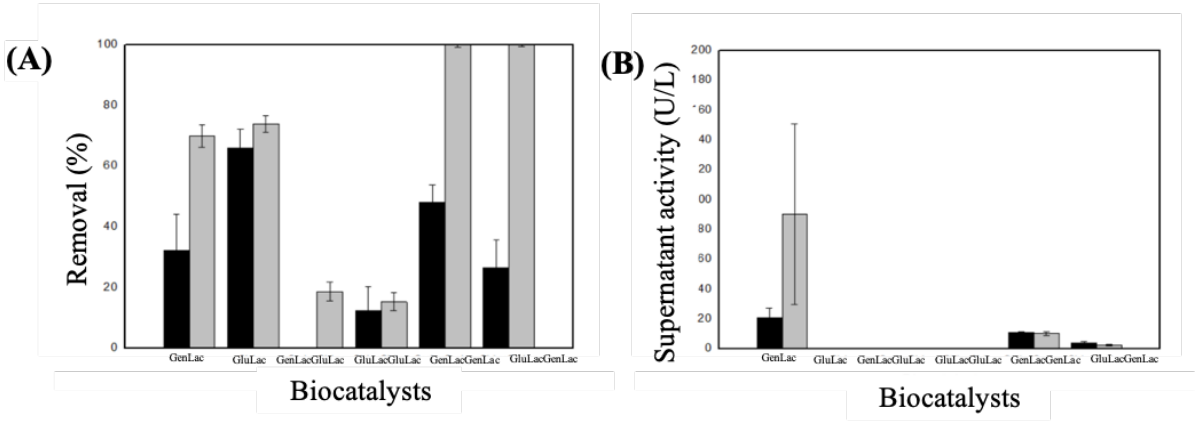
Regarding the operational stability of the single-layer biocatalysts (Figure 14B), it can be noticed that both biocatalysts maintained their initial activity after five consecutive cycles of ABTS oxidation. This indicates that even when submitted to a continuously-reprocessed solution, the enzyme molecules did not suffer significant conformational changes induced by the surrounding environment[54]. In contrast, the literature reported a 20% and 10.7 % loss of initial activity after the fifth cycle when laccase was immobilized onto genipin-activated chitosan (MA et al., 2018) and onto glutaraldehyde-activated chitosan (BILAL et al., 2019), respectively. This loss of activity could be a result of enzyme denaturation due to

conformational changes (MA et al., 2018). Regarding operational stability, the two-layer biocatalyst GenLacGluLac remained fully active after five consecutive cycles of reaction, while GluLacGluLac lost around 20% of its initial activity in the first cycle, with this value remaining constant in the following cycles. Both laccase layers in GluLacGluLac were prepared with glutaraldehyde, which can still remain chemically reactive and generate new laccase-support interactions during its use. This can lead to a negative effect on activity cycles (BARBOSA et al., 2013). On the other hand, the two-layer biocatalysts whose second layers were prepared with genipin as a crosslinker (GenLacGenLac and GluLacGenLac) showed no significant decays in activity after five cycles (Figure 14F). This appears to suggest that the catalytic site conformation was preserved, since the biocatalysts maintained their activity during the cycles (FLORES et al., 2019). In practical terms, biocatalyst reuse leads to a reduction in operational process costs, which is a highly desirable advantage of immobilized biocatalysts when compared to free enzymes (FLORES et al., 2019; SHELDON; VAN PELT, 2013).

4.4.4 Oxidation of selected trace organic contaminants

Figure 15 shows the elimination efficiency of the multilayer biocatalysts towards acetaminophen and mefenamic acid. Laccases mediate the single-electron oxidation of these pollutants that results in the formation of radicals as primary products (TOUAHAR et al., 2014). The radicals generated can couple with each other to form oligomers by covalent bonding of the two-parent molecules with one hydrogen atom that is eliminated from each molecule (TOUAHAR et al., 2014). These free radicals can then interact with electron donor groups present in other compounds, as well as with electron-donating functional groups such as amines (TOUAHAR et al., 2014).

Figure 15 – (A) Percentage of removal of acetaminophen (black columns) and mefenamic acid (grey columns) after a contact time of 24h. (B) Supernatant activity (free laccase; U/L) after reaction.



Source: elaborated by the author.

On most occasions, the use of the two-layer biocatalysts in this work did not result in an increase in acetaminophen removal (Figure 15A). Furthermore, when the second laccase layer was prepared with glutaraldehyde (GenLacGluLac and GluLacGluLac), almost no acetaminophen was removed. GluLacGenLac (26.6%) presented a considerable decrease in the removal performance compared to GluLac (66.1 %). In contrast, when the second laccase layer was prepared with genipin, a slight improvement was observed in the reactions catalyzed by GenLacGenLac (48.2 %) compared to GenLac (32.2%). It is important to note that all the double-layer biocatalysts in which glutaraldehyde was used (GenLacGluLac, GluLacGenLac, and GluLacGluLac) showed a decrease in removal performance. This was especially the case for the biocatalysts possessing glutaraldehyde-based second layers (GenLacGluLac and GluLacGluLac). This trend too can then be attributed to the fact that glutaraldehyde is still able to generate new laccase-support interactions during the process, since it maintains its chemical reactivity for long periods of time following activation (BARBOSA et al., 2013). Also, these new interactions are prone to affect more the two-layer biocatalysts, once more interactions take place in them than in the single-layer systems.

It is important to mention, however, that some of the biocatalysts prepared in this work still showed better performance than those reported by Hachi et al. (2017), who obtained a maximum removal of 25% of acetaminophen at pH 7.0 after 24h of reaction. Also, laccase is reported to degrade acetaminophen, delivering 94% of maximum removal in 4h at pH 7.0 (RATANAPONGLEKA; PUNBUT, 2018), and 90% after 2h of treatment (GARCÍA-

MORALES et al., 2018). The levels of acetaminophen removal shown Figure 15A were not expected since compounds like acetaminophen are prone to oxidative attack due to the strong electron-donating groups (EDG) present (ARCA-RAMOS et al., 2016); however, they could be explained due to the slower substrate diffusion within the laccase layers (BORTONE; FIDALEO; MORESI, 2014; SANTOS et al., 2015).

The biocatalysts produced in this study presented higher removal of the non-phenolic pharmaceutical active compound (PhACs) mefenamic acid than of the phenolic PhACs acetaminophen (Figure 15A). This could be related with the different substrate binding pocket in laccase (ERNST et al., 2018). After immobilization a more exposed position to mefenamic acid binding pocket my occur compared with acetaminophen binding pocket. The high hydrophobicity of mefenamic acid, poor solubility in water and moderate solubility in polar protic solvents can also explain this behavior owing to its free vinyl groups, which allows the aniline compound to be more available for enzymatic attack (ARCA-RAMOS et al., 2016; MUDALIP et al., 2019). Although the double-layer biocatalysts prepared with genipin as the second layer presented lower activity than those prepared with glutaraldehyde (Table 7), the levels of mefenamic acid removal followed a different behavior. The biocatalysts GenLacGenLac and GluLacGenLac were able to remove 100% of the initial concentration of mefenamic acid. In contrast, the two-layer biocatalysts prepared with glutaraldehyde in the second layer exhibited degradation rates of 18.7 ± 3.08 % and 15.4 ± 2.96 for GenLacGluLac and GluLacGluLac, respectively. It is not uncommon for the activity in the target substrate to be very different from its physiological activity. This is a phenomenon that can be caused by biocatalyst hyperactivation towards a particular substrate owing to a more active form of the enzyme resulting from a particular immobilization method (ARCA-RAMOS et al., 2016). Also, the results obtained for mefenamic acid degradation are in accordance with literature (KUMAR, V. V.; CABANA, 2016; PEREIRA et al., 2020). For instance, magnetic laccase CLEAs (1000 U/L) were able to almost completely remove (99%) mefenamic acid in a mixture of pharmaceuticals (100 µg/L of each) after 6h (KUMAR, V. V.; CABANA, 2016), while free laccases (730 U/L) removed 100% of this compound (20 mg/L) after 4h(PEREIRA et al., 2020).

During the immobilization process, some of the physicochemical properties in the enzyme surroundings may be altered, creating a more hydrophobic or hydrophilic environment that, in turn, can generate a partitioning effect of different compounds away or towards the enzyme (RODRIGUES, R. C. et al., 2013). Chitosan activated with genipin shows more hydrophilicity than chitosan crosslinked with glutaraldehyde, and an increase in

the activation concentration of both crosslinkers will cause a decrease in the support hydrophilicity (DU et al., 2020). This decay can justify the fact that all biocatalysts showed better performance in the degradation of mefenamic acid, which is a hydrophobic compound (ARCA-RAMOS et al., 2016). The high hydrophobicity of mefenamic acid could then generate a concentration gradient of the substrate onto the surface of the biocatalysts, promoting their adsorption onto the biocatalysts due to the presence of free vinyl groups and making it more available for enzymatic attack (ARCA-RAMOS et al., 2016).

A degree of enzyme leakage was observed in biocatalysts prepared using genipin (GenLac, GenLacGenLac, and GluLacGenLac) after 24h of reaction (Figure 15B). These results show that some laccases could become immobilized by adsorption, most likely due to parallel reactions between genipin molecules. This, in turn, can lead to fewer groups available for laccase immobilization by covalent attachment (BUTLER; NG; PUDNEY, 2003). Even though the performance of the biocatalysts concerning the degradations of PhACs was lower than expected, laccase-based processes are still advantageous in which they present a low risk of toxic product generation. This renders the use of this enzyme in its insolubilized form very promising for water treatment if compared to conventional wastewater treatment plants (BA et al., 2014).

4.5 Conclusion

The layer-by-layer laccase immobilization using genipin and glutaraldehyde produced biocatalysts with different activities and stabilities. The effect of the coating process was positive under some conditions tested, and, in general, the multilayer biocatalysts were less stable than their single-layer counterparts. The two-layer biocatalysts possessing genipin-based second layers presented lower activity than those that were glutaraldehyde-based. However, these biocatalysts still presented a better performance in the removal of PhACs. Based on the results obtained, it can be concluded that chitosan activation with different crosslinkers, coating processes, and immobilization layers produced biocatalysts with different specificities. Despite these differences, the multilayer strategy seems valid in the production of potent biocatalysts for the degradation of phenolic compounds.

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4.7 References

ADDORISIO, V.; SANNINO, F.; MATEO, C.; GUISÁN, J. M. Oxidation of phenyl compounds using strongly stable immobilized-stabilized laccase from Trametes versicolor. **Process Biochemistry**, [*s. l.*], v. 48, n. 8, p. 1174–1180, 2013.

ALI, A.; AHMED, S. A review on chitosan and its nanocomposites in drug delivery. **International Journal of Biological Macromolecules**, [*s. l.*], v. 109, p. 273–286, 2018.

ARANA-PEÑA, S. Coimmobilization of different lipases: Simple layer by layer enzyme spatial ordering. **International Journal of Biological Macromolecules**, [*s. l.*], v. 145, p. 856–864, 2020a.

ARANA-PEÑA, S.; RIOS, N. S.; MENDEZ-SANCHEZ, C.; LOKHA, Y.; GONÇALVES, L. R. B.; FERNÁNDEZ-LAFUENTE, R. Use of polyethylenimine to produce immobilized lipase multilayers biocatalysts with very high volumetric activity using octyl-agarose beads: Avoiding enzyme release during multilayer production. **Enzyme and Microbial Technology**, [*s. l.*], v. 137, p. 109535, 2020, 2019.

ARCA-RAMOS, A. Recyclable cross-linked laccase aggregates coupled to magnetic silica microbeads for elimination of pharmaceuticals from municipal wastewater. **Environmental Science and Pollution Research**, [*s. l.*], v. 23, n. 9, p. 8929–8939, 2016.

AVCU, E. Electrophoretic deposition of chitosan-based composite coatings for biomedical applications: A review. **Progress in Materials Science**, [*s. l.*], v. 103, p. 69–108, 2019.

BA, S.; HAROUNE, L.; CRUZ-MORATÓ, C.; JACQUET, C.; TOUAHAR, I. E.; BELLENGER, J.; LEGAULT, C. Y.; JONES, J. P.; CABANA, H. Synthesis and characterization of combined cross-linked laccase and tyrosinase aggregates transforming acetaminophen as a model phenolic compound in wastewaters. **Science of The Total Environment**, [*s. l.*], v. 487, n. 1, p. 748–755, 2014.

BARBOSA, O.; ORTIZ, C.; BERENGUER-MURCIA, Á.; TORRES, R.; RODRIGUES, R. C.; FERNANDEZ-LAFUENTE, R. Glutaraldehyde in bio-catalysts design: a useful crosslinker and a versatile tool in enzyme immobilization. **RSC Adv.**, [*s. l.*], v. 4, n. 4, p. 1583–1600, 2014.

BARBOSA, O.; TORRES, R.; ORTIZ, C.; BERENGUER-MURCIA, A.; RODRIGUES, R. C.; FERNÁNDEZ-LAFUENTE, R. **Heterofunctional supports in enzyme immobilization: From traditional immobilization protocols to opportunities in tuning enzyme properties**. [*s. l.*], 2013.

BELLÉ, A. S.; FERRANDI, E. E.; RIVA, S.; MONTI, D. Efficient enzyme-assisted extraction of genipin from genipap (*Genipa americana L.*) and its application as a crosslinker for chitosan gels. **Food Chemistry**, [*s. l.*], v. 246, p. 266–274, 2018.

BEZERRA, C. S. Enzyme immobilization onto renewable polymeric matrixes: Past, present, and future trends. **Journal of Applied Polymer Science**, [*s. l.*], v. 132, n. 26, p. n/a-n/a, 2015.

BILAL, M. Immobilization of fungal laccase on glutaraldehyde cross-linked chitosan beads and its bio-catalytic potential to degrade bisphenol A. **Biocatalysis and Agricultural Biotechnology**, [*s. l.*], v. 19, p. 101174, 2019.

BORTONE, N.; FIDALEO, M.; MORESI, M. Internal and external mass transfer limitations on the activity of immobilised acid urease derivatives differing in enzyme loading. **Biochemical Engineering Journal**, [*s. l.*], v. 82, p. 22–33, 2014.

BOUDRANT, J.; WOODLEY, J. M.; FERNANDEZ-LAFUENTE, R. Parameters necessary to define an immobilized enzyme preparation. **Process Biochemistry**, [*s. l.*], v. 90, p. 66–80, 2020.

BRUGNARI, T.; PEREIRA, M. G.; BUBNA, G. A.; FREITAS, E. N.; CONTATO, A. G.; CORRÊA, R. C. G.; CASTOLDI, R.; SOUZA, C. G. M.; POLIZELI, M. L. T. M.; BRACHT, A.; PERALTA, R. M. A highly reusable MANAE-agarose-immobilized Pleurotus ostreatus laccase for degradation of bisphenol A. **Science of the Total Environment**, [*s. l.*], v. 634, p. 1346–1351, 2018.

BUTLER, M. F.; NG, Y.; PUDNEY, P. D. A. Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin. **Journal of Polymer Science Part A: Polymer Chemistry**, [*s. l.*], v. 41, n. 24, p. 3941–3953, 2003.

CARUSO, F.; SCHÜLER, C. Enzyme multilayers on colloid particles: Assembly, stability, and enzymatic activity. **Langmuir**, [*s. l.*], v. 16, n. 24, p. 9595–9603, 2000.

CHAGAS, P. M. B. Immobilized soybean hull peroxidase for the oxidation of phenolic compounds in coffee processing wastewater. **International Journal of Biological Macromolecules**, [*s. l.*], v. 81, p. 568–575, 2015.

CHRISTWARDANA, M. Combination of physico-chemical entrapment and crosslinking of low activity laccase-based biocathode on carboxylated carbon nanotube for increasing biofuel cell performance. **Enzyme and Microbial Technology**, [*s. l.*], v. 106, p. 1–10, 2017.

CRESTINI, C.; PERAZZINI, R.; SALADINO, R. Oxidative functionalisation of lignin by layer-by-layer immobilised laccases and laccase microcapsules. **Applied Catalysis A: General**, [*s. l.*], v. 372, n. 2, p. 115–123, 2010.

CUI, C. Genipin Cross-Linked Glucose Oxidase and Catalase Multi-enzyme for Gluconic Acid Synthesis. **Applied Biochemistry and Biotechnology**, [*s. l.*], v. 181, n. 2, p. 526–535, 2017.

DAI, J.; MUMPER, R. J. Plant Phenolics: Extraction, analysis and their antioxidant and anticancer properties. **Molecules**, [*s. l.*], v. 15, n. 10, p. 7313–7352, 2010.

DARONCH, N.; KELBERT, M.; PEREIRA, C. S.; ARAÚJO, P. H. H.; OLIVEIRA, D. Elucidating the choice for a precise matrix for laccase immobilization: A review. **Chemical Engineering Journal**, [*s. l.*], v. 397, p. 125506, 2020.

DELMAR, K.; BIANCO-PELED, H. The dramatic effect of small pH changes on the properties of chitosan hydrogels crosslinked with genipin. **Carbohydrate Polymers**, [*s. l.*], v. 127, p. 28–37, 2015.

DU, J. R.; HSU, L. H.; XIAO, E. S.; GUO, X.; ZHANG, Y.; FENG, X. Using genipin as a “green” crosslinker to fabricate chitosan membranes for pervaporative dehydration of isopropanol. **Separation and Purification Technology**, [*s. l.*], v. 244, p. 116-843, 2020.

ERNST, H. A.; JORGENSEN, L. J.; BUKH, C.; PIONTEK, K.; PLATTNER, D. A.; OSTERGAARD, L. H.; LARSEN, S.; BJERRUM, M. J. A comparative structural analysis of the surface properties of asco-laccases. **PLOS ONE**, [*s. l.*], v. 13, n. 11, p. 1-27, 2018.

FERNÁNDEZ-FERNÁNDEZ, M.; SANROMÁN, M. Á.; MOLDES, D. Recent developments and applications of immobilized laccase. **Biotechnology Advances**, [*s. l.*], v. 31, n. 8, p. 1808–1825, 2013.

FLORES, E. E. E.; CARDOSO, F. D.; SIQUEIRA, L. B.; RICARDI, N. C.; COSTA, T. H.; RODRIGUES, R. C.; KLEIN, M. P.; HERTZ, P. F. Influence of reaction parameters in the polymerization between genipin and chitosan for enzyme immobilization. **Process Biochemistry**, [*s. l.*], v. 84, n. June, p. 73–80, 2019.

GARCÍA-MORALES, R. Biotransformation of emerging pollutants in groundwater by laccase from P. sanguineus CS43 immobilized onto titania nanoparticles. **Journal of Environmental Chemical Engineering**, [*s. l.*], v. 6, n. 1, p. 710–717, 2018.

HACHI, M. Removal of acetaminophen and carbamazepine in single and binary systems with immobilized laccase from Trametes hirsuta. **Biocatalysis and Biotransformation**, [*s. l.*], v. 35, n. 1, p. 51–62, 2017.

HAROUNE, L. Evaluation of the efficiency of Trametes hirsuta for the removal of multiple pharmaceutical compounds under low concentrations relevant to the environment. **Bioresource Technology**, [*s. l.*], v. 171, n. 1, p. 199–202, 2014.

HOARAU, M.; BADIEYAN, S.; MARSH, E. N. G. Immobilized enzymes: understanding enzyme – surface interactions at the molecular level. **Organic & Biomolecular Chemistry**, [*s. l.*], v. 15, n. 45, p. 9539–9551, 2017.

KILDEEVA, N. R.; PERMINOV, P. A.; VLADIMIROV, L. V.; NOVIKOV, V. V.; MIKHAILOV, S. N. About mechanism of chitosan cross-linking with glutaraldehyde. **Russian Journal of Bioorganic Chemistry**, [*s. l.*], v. 35, n. 3, p. 360–369, 2009.

KILINÇ, A.; ÖNAL, S.; TELEFONCU, A. Stabilization of papain by modification with chitosan. **Turkish Journal of Chemistry**, [*s. l.*], v. 26, n. 3, p. 311–316, 2002.

KLEIN, M. Chitosan crosslinked with genipin as support matrix for application in food process: Support characterization and β- d -galactosidase immobilization. **Carbohydrate Polymers**, [*s. l.*], v. 137, p. 184–190, 2016.

KUMAR, V. V.; CABANA, H. Towards high potential magnetic biocatalysts for on-demand elimination of pharmaceuticals. **Bioresource Technology**, [*s. l.*], v. 200, p. 81–89, 2016.

LEE, J.; LEE, D.; JEONG, C. Gardenia jasminoides Ellis ethanol extract and its constituents reduce the risks of gastritis and reverse gastric lesions in rats. **Food and Chemical Toxicology**, [*s. l.*], v. 47, n. 6, p. 1127–1131, 2009.

MA, H.; MENG, G.; CUI, B.; SI, J.; DAI, Y. Chitosan crosslinked with genipin as supporting matrix for biodegradation of synthetic dyes: Laccase immobilization and characterization. **Chemical Engineering Research and Design**, [*s. l.*], v. 132, n. 35, p. 664–676, 2018.

MANICKAM, B.; SREEDHARAN, R.; ELUMALAI, M. 'Genipin' – The natural water soluble cross-linking agent and its importance in the modified drug delivery systems: An overview. **Current Drug Delivery**, [*s. l.*], v. 11, n. 1, p. 139–145, 2014.

MARIA, P. Catalytic stability of turnip peroxidase in free and immobilized form on chitosan beads. **International Journal of Current Microbiology and Applied Sciences**, [*s. l.*], v. 3, n. 11, p. 576–595, 2014.

MATEO, C. Glyoxyl agarose: A fully inert and hydrophilic support for immobilization and high stabilization of proteins. **Enzyme and Microbial Technology**, [*s. l.*], v. 39, n. 2, p. 274–280, 2006.

MENDES, A. A.; DE CASTRO, H. F.; ANDRADE, G. S. S.; TARDIOLI, P. W.; GIORDANO, R. L. C. Preparation and application of epoxy-chitosan/alginate support in the immobilization of microbial lipases by covalent attachment. **Reactive and Functional Polymers**, [*s. l.*], v. 73, n. 1, p. 160–167, 2013.

MOLL, R.; DERRY, S.; MOORE, R. A.; MCQUAY, H. J. Single dose oral mefenamic acid for acute postoperative pain in adults. **Cochrane Database of Systematic Reviews**, [*s. l.*], n. 3, 2011.

MORSI, R.; BILAL, M.; IQBAL, H. M. N.; ASHRAF, S. Laccases and peroxidases: The smart, greener and futuristic biocatalytic tools to mitigate recalcitrant emerging pollutants. **Science of The Total Environment**, [*s. l.*], v. 714, p. 136-572, 2020.

MUDALIP, S. K.; ABU BAKAR, M. R.; JAMAL, P.; ADAM, F.; ALAM, Z. M. Molecular recognition and solubility of mefenamic acid in water. **Asian Journal of Chemistry**, [*s. l.*], v. 28, n. 4, p. 853–858, 2016.

MUDALIP, S. K.; ABU BAKAR, M. R.; JAMAL, P.; ADAM, F. Prediction of mefenamic acid solubility and molecular interaction energies in different classes of organic solvents and water. **Ind. Eng. Chem.Res**, [*s. l.*], v. 58, n. 2, p. 762-770, 2019.

MUZZARELLI, R. A. A.; EL MEHTEDI, M.; BOTTEGONI, C.; GIGANTE, A. Physical properties imparted by genipin to chitosan for tissue regeneration with human stem cells: A review. **International Journal of Biological Macromolecules**, [*s. l.*], v. 93, p. 1366–1381, 2016.

NAGHDI, M. Pinewood nanobiochar: A unique carrier for the immobilization of crude laccase by covalent bonding. **International Journal of Biological Macromolecules**, [*s. l.*], v. 115, p. 563–571, 2018.

NGUYEN, H. H.; KIM, M. An overview of techniques in enzyme immobilization. **Applied Science and Convergence Technology**, [*s. l.*], v. 26, n. 6, p. 157–163, 2017.

NUNES, C. Chitosan–caffeic acid–genipin films presenting enhanced antioxidant activity and stability in acidic media. **Carbohydrate Polymers**, [*s. l.*], v. 91, n. 1, p. 236–243, 2013.

OLIVEIRA, G. Feasibility of chitosan crosslinked with genipin as biocoating for cellulose-based materials. **Carbohydrate Polymers**, [*s. l.*], v. 242, p. 116429, 2020.

PEREIRA, C. S. Potential of enzymatic process as an innovative technology to remove anticancer drugs in wastewater. **Applied Microbiology and Biotechnology**, [*s. l.*], v. 104, n. 1, p. 23–31, 2020.

PINHEIRO, B. B.; RIOS, N. S.; RODRÍGUEZ AGUADO, E.; FERNANDEZ-LAFUENTE, R.; FREIRE, T. M.; FECHINE, P. B. A.; DOS SANTOS, J. C. S.; GONÇALVES, L. R. B. Chitosan activated with divinyl sulfone: a new heterofunctional support for enzyme immobilization. Application in the immobilization of lipase B from Candida antarctica. **International Journal of Biological Macromolecules**, [*s. l.*], v. 130, p. 798–809, 2019.

RATANAPONGLEKA, K.; PUNBUT, S. Removal of acetaminophen in water by laccase immobilized in barium alginate. **Environmental Technology**, [*s. l.*], v. 39, n. 3, p. 336–345, 2018.

RIOS, N.; ARANA-PEÑA, S.; MENDEZ-SANCHEZ, C.; LOKHA, Y.; CORTES-CORBERAN, V.; GONÇALVES, L. R. B.; FERNANDEZ-LAFUENTE, R. Increasing the enzyme loading capacity of porous supports by a layer-by-layer immobilization strategy using PEI as glue. **Catalysts**, [*s. l.*], v. 9, n. 7, p. 576, 2019.

RODRIGUES, R. C. Modifying enzyme activity and selectivity by immobilization. **Chem. Soc. Rev.**, [*s. l.*], v. 42, n. 15, p. 6290–6307, 2013.

RODRÍGUEZ COUTO, S.; TOCA HERRERA, J. L. **Industrial and biotechnological applications of laccases: A review**. [*s. l.*], 2006.

SANTOS, J. C. S.; BARBOSA, O.; ORTIZ, C.; BERENGUER-MURCIA, A.; RODRIGUES, R. C.; FERNANDEZ-LAFUENTE, R. Importance of the Support Properties for Immobilization or Purification of Enzymes. **ChemCatChem**, [*s. l.*], v. 7, n. 16, p. 2413–2432, 2015.

SECUNDO, F. Conformational changes of enzymes upon immobilisation. **Chemical Society Reviews**, [*s. l.*], v. 42, n. 15, p. 6250, 2013.

SEMERDZHIEVA, V.; RAYKOVA, R.; MARINKOVA, D.; YANEVA, S. Chernev g and iliev i. layer-by-layer assembly of enzymes and nanoparticles onto cellulose support. **Journal of Biosensors & Bioelectronics**, [*s. l.*], v. 09, n. 04, 2018.

SHELDON, R. A.; VAN PELT, S. Enzyme immobilisation in biocatalysis: why, what and how. **Chem. Soc. Rev.**, [*s. l.*], v. 42, n. 15, p. 6223–6235, 2013.

SILVA, J. A.; MACEDO, G. P.; RODRIGUES, D. S.; GIORDANO, R. L. C.; GONÇALVES, L. R. B. Immobilization of *Candida antarctica* lipase B by covalent attachment on chitosan-based hydrogels using different support activation strategies. **Biochemical Engineering Journal**, [*s. l.*], v. 60, p. 16–24, 2012.

SMITH, P. K. Measurement of protein using bicinchoninic acid. **Analytical Biochemistry**, [*s. l.*], v. 150, n. 1, p. 76–85, 1985.

SUNG, H. Stability of a biological tissue fixed with a naturally occurring crosslinking agent (genipin). **Journal of Biomedical Materials Research**, [*s. l.*], v. 55, n. 4, p. 538–546, 2001.

TACIAS-PASCACIO, V. G.; GARCÍA-PARRA, E.; VELA-GUTIÉRREZ, G.; VIRGEN-ORTIZ, J. J.; BERENGUER-MURCIA, Á.; ALCÁNTARA, A. R.; FERNANDEZ-LAFUENTE, R. Genipin as an emergent tool in the design of biocatalysts: mechanism of reaction and applications. **Catalysts**, [*s. l.*], v. 9, n. 12, p. 1035, 2019.

TAVARES, L. Effect of deacetylation degree of chitosan on rheological properties and physical chemical characteristics of genipin-crosslinked chitosan beads. **Food Hydrocolloids**, [*s. l.*], v. 106, p. 105876, 2020.

TOMIMATSU, Y. Physical chemical observations on the α-chymotrypsin glutaraldehyde system during formation of an insoluble derivative. **Journal of Colloid and Interface Science**, [*s. l.*], v. 36, n. 1, p. 51–64, 1971.

TOUAHAR, I. E.; HAROUNE, L.; BA, S.; BELLENGER, J.; CABANA, H. Characterization of combined cross-linked enzyme aggregates from laccase, versatile peroxidase and glucose oxidase, and their utilization for the elimination of pharmaceuticals. **Science of The Total Environment**, [*s. l.*], v. 481, n. 1, p. 90–99, 2014.

VIRGEN-ORTÍZ, J. J.; SANTOS, J. C. S.; BERENGUER-MURCIA, Á.; BARBOSA, O.; RODRIGUES, R. C.; FERNANDEZ-LAFUENTE, R. **Polyethylenimine: A very useful ionic polymer in the design of immobilized enzyme biocatalysts**. [*s. l.*], 2017.

WANG, Q.; JIANG, J.; XIONG, Y. L. Genipin-aided protein cross-linking to modify structural and rheological properties of emulsion-filled hempseed protein hydrogels. **Journal of Agricultural and Food Chemistry**, [*s. l.*], v. 67, n. 46, p. 12895–12903, 2019.

WOODLEY, J. M. New opportunities for biocatalysis: making pharmaceutical processes greener. **Trends in Biotechnology**, [*s. l.*], v. 26, n. 6, p. 321–327, 2008.

XU, B.; ZHAN, G.; XU, B.; DU, H.; LUO, H.; WANG, T.; ZHAN, C.; YANG, Y. Degradation of acetaminophen in aqueous solution by UV and UV-activated sludge processes. **Water Science and Technology**, [*s. l.*], v. 78, n. 10, p. 2088–2095, 2018.

ZHANG, X.; HUA, M.; LV, L.; PAN, B. Ionic Polymer-Coated Laccase with High Activity and Enhanced Stability: Application in the Decolourisation of Water Containing AO7. **Scientific Reports**, [*s. l.*], v. 5, n. 1, p. 8253, 2015. Disponível em: https://doi.org/10.1038/srep08253

ZHENG, F. Immobilization of laccase onto chitosan beads to enhance its capability to degrade synthetic dyes. **International Biodeterioration & Biodegradation**, [*s. l.*], v. 110, n. 4, p. 69–78, 2016.

Chapter 5

Final considarations

5 FINAL CONSIDERATIONS

In this study, some strategies for production of biocatalysts through different techniques of immobilization to eliminate micropollutants presented in wastewaters were performed. To carry out this project, it was kept in mind the strategy of laccase immobilization once laccase-based biocatalysts have gained attention to achieve this purpose. Different strategies of immobilization were performed: adsorption on modified agarose and immobilization on heterofunctional supports.

The chemical amination of laccase using different concentration of EDAC has proved to be a valid strategy to produce protein surfaces enriched with more reactive groups. Aminated laccase immobilized by ionic adsorption on cationic and anionic supports showed better immobilization parameters as more amino groups were introduced. The same tendency was observed in thermal stability performance once chemical amination process allows the introduction of amino groups on the enzyme surface, which might enhance enzyme rigidity and stability. However, these preparations presented lower thermal stability than free aminated laccases. As future perspectives for this part of the work we suggest the production and characterization of CLEA's with aminated laccases using strategies to achieve CLEA's with space beteween laccases. The production of laccase aminated CLEA's may provide high active and stable biocatalysts able to good results in reactor's application.

The preparations immobilized by layer-by-layer onto chitosan activated with genipin or glutaraldehyde were performed to enhance the loading capacity of the support. This technical of laccase immobilization using genipin and glutaraldehyde produced biocatalysts with different activities and stabilities. However, the addition of a second layer did not always produce a more active biocatalyst and the multilayer biocatalysts were less stable than their single-layer counterparts. The application of the biocatalysts produced in the degradation of acetaminophen and mefenamic acid showed different performances. The two-layer biocatalysts possessing genipin-based second layers presented better degradation of contaminants than those that were glutaraldehyde-based even being less active with ABTS substrate. Based on the results obtained, the strategy used to immobilize laccase produced biocatalysts with different specificities. Despite these differences, the multilayer strategy seems valid in the production of potent biocatalysts for the degradation of phenolic compounds. As future perspectives for this second part of the work we suggest the study of

different conditions of chitosan activation with genipin as well as different conditions of laccase immobilization and applications.